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TOXIC EFFECT OF IRRADIATED MEDIUM ON MAMMALIAN CELLS IN TISSUE CULTURE

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Toxic Effect of Irradiated Medium on Mammalian Cells in Tissue Culture" submitted by David Roback in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Indirect radiation damage to cells mediated by long-lived species formed in the medium has been studied. Growth characteristics of mouse leukemic lymphoblasts (L5178Y) in tissue culture were used as the index of damage.

Growth inhibition and loss of viability were seen after incubation in irradiated medium. The effect increased with dose to medium and time of incubation, and decreased as the interval between medium irradiation and inoculation increased. Cytotoxicity was also diminished at low incubation temperatures. Incubation in irradiated PBS was significantly more damaging than in irradiated medium, and inoculation time dependence was not seen. The serum in medium protected through enzymatic or chemical reaction with the cytotoxic agent.

Catalase added 1 minute before inoculation offered total protection in medium, but only limited protection in PBS. The cytotoxic effect was duplicated when cells were incubated in medium containing reagent hydrogen peroxide at the concentration corresponding to its measured radiolytic yield. Cytotoxic action in irradiated medium was therefore attributed to H_2O_2 formed in the radiolysis of medium. However, the effect was not reproducible in PBS containing H_2O_2. Measurements of H_2O_2 yields in irradiated chloride solutions suggested the involvement of chlorine radical products in the cytotoxic action of irradiated PBS.

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INTRODUCTION TO RADIATION BIOLOGY

1.1 IMMEDIATE EVENTS

1.1.1 Interaction of Ionizing Radiation with Matter

Ionizing radiation refers to those radiations, electromagnetic or particulate, which deposit their energy by ejection of atomically bound electrons in the absorbing matter. The primary radiation beam is attenuated exponentially. The exponential coefficient, which is a function of the atomic composition of the absorbing material and the energy of the primary beam, is the sum of the coefficients of separate absorption processes. In the case of electromagnetic radiation (x-rays and γ-rays), with which we will deal exclusively from here, the important processes are the photoelectric effect, the Compton effect, and pair production. These processes are explained in detail elsewhere (Evans 1968).

Briefly, the photoelectric effect predominates at low energies (less than 100 keV) and high atomic number (Z) material, and involves the absorption of an incident photon and subsequent ejection of an atomic electron. Compton scattering predominates at intermediate energies (0.1 to 1.0 MeV). In this process a collision between an incident photon and a "free" atomic electron results in a scattered photon and recoil electron. The energies and directions of scatter

of the collision products depend upon the incident photon energy. The scattered photon may eventually be absorbed by Compton and photoelectric processes. At energies greater than twice the electron rest mass (1.02 MeV) the incident photon is subject to the process of pair production, in which an electron-positron pair is spontaneously formed. This process is not important at energies less than 4 MeV and in low Z material, e.g. water or aqueous systems.

The initial deposition of energy is non-selective. It does not depend on the chemical structure of the absorbing molecule, in contrast to the non-ionizing electromagnetic radiations, microwave to vacuum ultra-violet.

These primary processes produce a spectrum of energetic scattered electrons which travel in tracks through the medium, losing their energy (or slowing down) through coulombic interactions with molecular electrons lying near the track. The proximity of approach determines the energy imparted to the molecule and secondary electron. Thus a knock-on collision will produce a fast secondary electron (a delta ray), while a glancing collision will impart only enough energy for molecular electronic excitation, or a few ionizations. Mozumder and Magee (1966) have classified secondary (and subsequent generation) electrons according to their energy and the sort of ionization patterns they produce.

A spur is a spherical region of several ionizations resulting from a secondary electron with energy less than 100 ev, produced by a glancing collision. Knock-on electrons are divided into those giving rise to blobs, short tracks and branch tracks. A blob, resembling a large spur, is produced by a knock-on electron with insufficient kinetic energy (between 100 ev and 500 ev) to escape its sibling hole. A short track is distinguished from a branch track by having overlapping spurs. The secondary electron giving rise to a branch track has kinetic energy greater than 5 keV. The three extra-spur entities are known collectively as delta-rays (figure 1).

The spatial distribution of energy transfer to the medium by a charged particle is described by the Linear Energy Transfer (LET). LET is defined (ICRU 1970) as " $L_\Delta = \left(\frac{dE}{dl}\right)_\Delta$, where dl is the distance traversed by the particle and dE is the mean energy loss due to collisions with energy transfers less than some specified Δ ".

The irradiation of water with ^{60}Co γ-rays produces primary electrons with initial energies about 1 MeV, as well as 10^2 to 10^3 ev δ-rays. The electron energy spectrum is therefore represented by a bimodal LET distribution. Since the track average LET, dominated by low LET fast electrons, and the dose average LET, dominated by δ-rays, differ by a

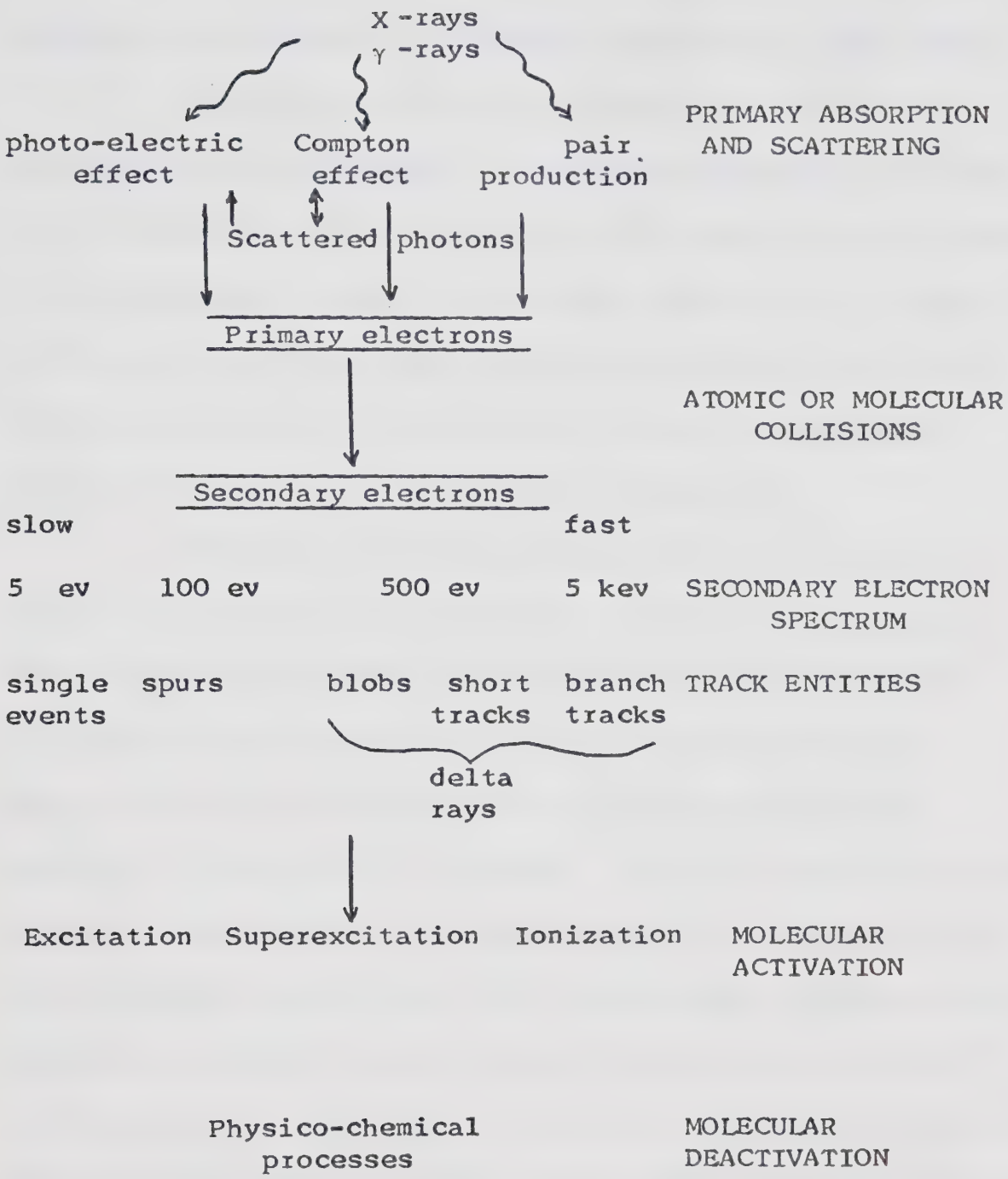


Figure 1. X-ray and γ-ray interaction in molecular medium

factor of about 30, the complete LET distribution of a radiation is necessary to specify its quality (ICRU 1970).

The distribution of electronic activation (i.e. excitation and ionization) in the medium depends on the LET of the ionizing particle, which in turn depends on its energy. A fast (low LET) primary or secondary electron will produce a straight track with well-spaced spurs while a slow (high LET) electron with hundreds of eV energy will describe a devious, densely ionizing path (see figure 2).

The amount of energy deposited in a unit mass of absorbing material by the ionizing radiation is the single most important parameter in determining radiation effects. The unit of absorbed dose (rad) is defined as 100 ergs imparted by ionizing particles per gram of irradiated material at the place of interest. This operative definition concerns itself with the absorbed dose in the material for the conditions under study. For irradiations of water-based biological systems, especially cell suspensions or enzyme solutions, it is convenient to determine the absorbed dose by measuring the chemical change in an aqueous chemical dosimeter exposed to identical irradiation conditions. The ferrous sulfate (or Fricke) dosimeter is most commonly used for biological radiation dosimetry. The radiation-induced chemical change is oxidation of ferrous ions in aqueous

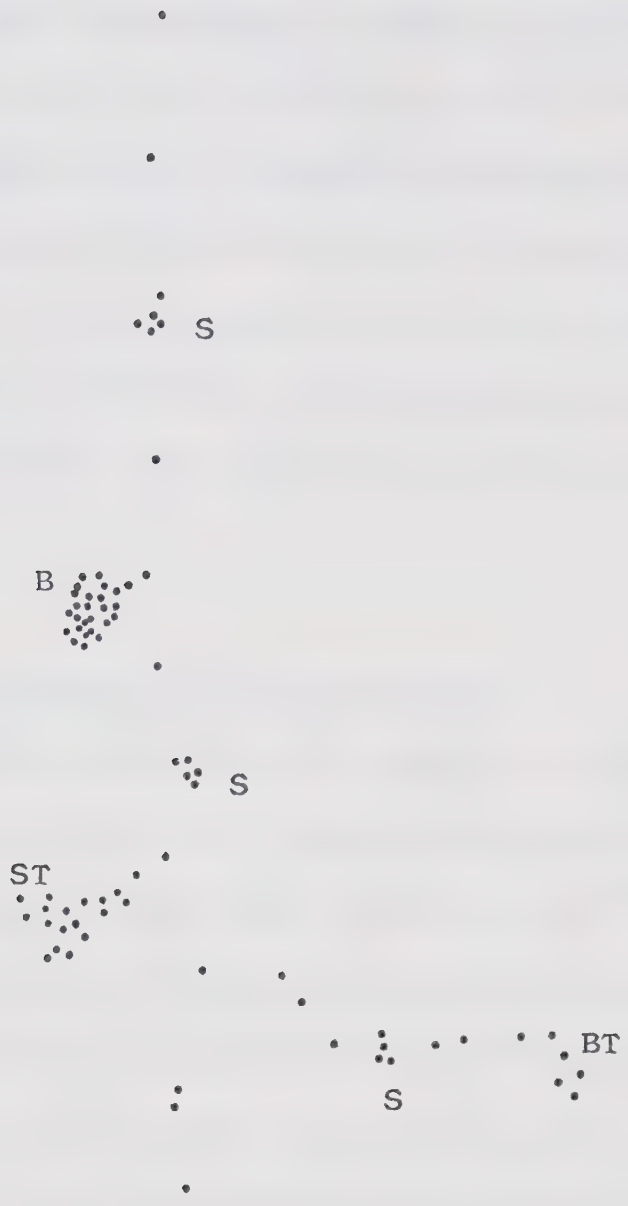


Figure 2. Track entities. Ionization pattern due to passage of an energetic primary electron through water. Spurs (S), blobs (B), short tracks (ST), and branch tracks (BT) are indicated.

solution to ferric ions. These can be measured spectrophotometrically, and the dosimeter calibrated against some primary dosimeter such as an ionization chamber. The Fricke dosimeter has a reproducible response to radiation dose in the krad to Mrad range, which is important in biological irradiations. It is also valuable for its post-irradiation stability, its energy and dose-rate independence, and because it is convenient and simple to use. (Fricke and Hart 1968).

1.1.2 Molecular Activation and Deactivation

The response of a molecular medium to ionizing radiation can be dealt with in three consecutive stages (figure 3). 1) The physical stage (less than 10^{-14} sec) involves the dissipation of radiant energy in the medium. 2) The physico-chemical stage (10^{-14} to 10^{-10} sec) consists of the molecular processes which restore the system to thermal equilibrium. 3) The chemical stage (10^{-10} to 10^{-3} sec) consists of reactions leading to chemical equilibrium in the medium. Since most of these reactions are diffusion controlled, we can separate the early track period consisting of reactions in the spur, from the uniform period, which follows the diffusion of reactive species away from the spur, and involves reactions with the solute in the bulk of the

- log t (sec)	Events	Stage
18	Mev electron traverses molecule	Physical
16	Vertical (electronic) excitation	
15	Time between successive ionizations by Mev electron	
14	Ion-moleculer reactions. Period of molecular vibrations. Fast dissociation	
12	Electron thermalizes	Physico-chemical
11	Relaxation time for dipole orientation in water	
10	Spur formation. Diffusion controlled reactions in spur	
8	Intra-spur reactions complete	Chemical
5	Radical - solute reactions	
4	Radical interspur diffusion time in MeV electron track	

Figure 3. Time scale in radiation chemistry.
(from Mozumder 1970, Spinks and Wood 1964)

medium. Clearly, the feature which distinguishes the two periods of the chemical stage is the spatial distribution of reactions in the medium.

Molecular processes occurring during the physical stage are a result of energy transfer from energetic electrons to the electronic system of the molecule. These include ionization, excitation, and superexcitation (figure 4). The relative yields of the various activated molecules depend on the energy spectrum of electrons in the medium (a property of the medium and the radiation), and the molecular activation cross-sections. Platzman (1967) has calculated primary yields in water by using the photon-molecule interaction spectrum to approximate the electron-molecule interaction cross-section. Yields calculated by this method, expressed as "G values" (molecules per 100 eV absorbed), are G(ionization) = 3.48, G(excitation) = 0.54, and G(superexcitation) = 0.92. Electronically activated molecules are generally in a vibrationally excited state, since their equilibrium interatomic bond distances are different from those in the ground state (Boag 1967). The Franck-Condon principle states that electronic transition occurs in a time much smaller than a vibrational period (10^{-16} sec vs. 10^{-13} sec), and accounts for the "vertical" electronic transition illustrated in figure 4b.

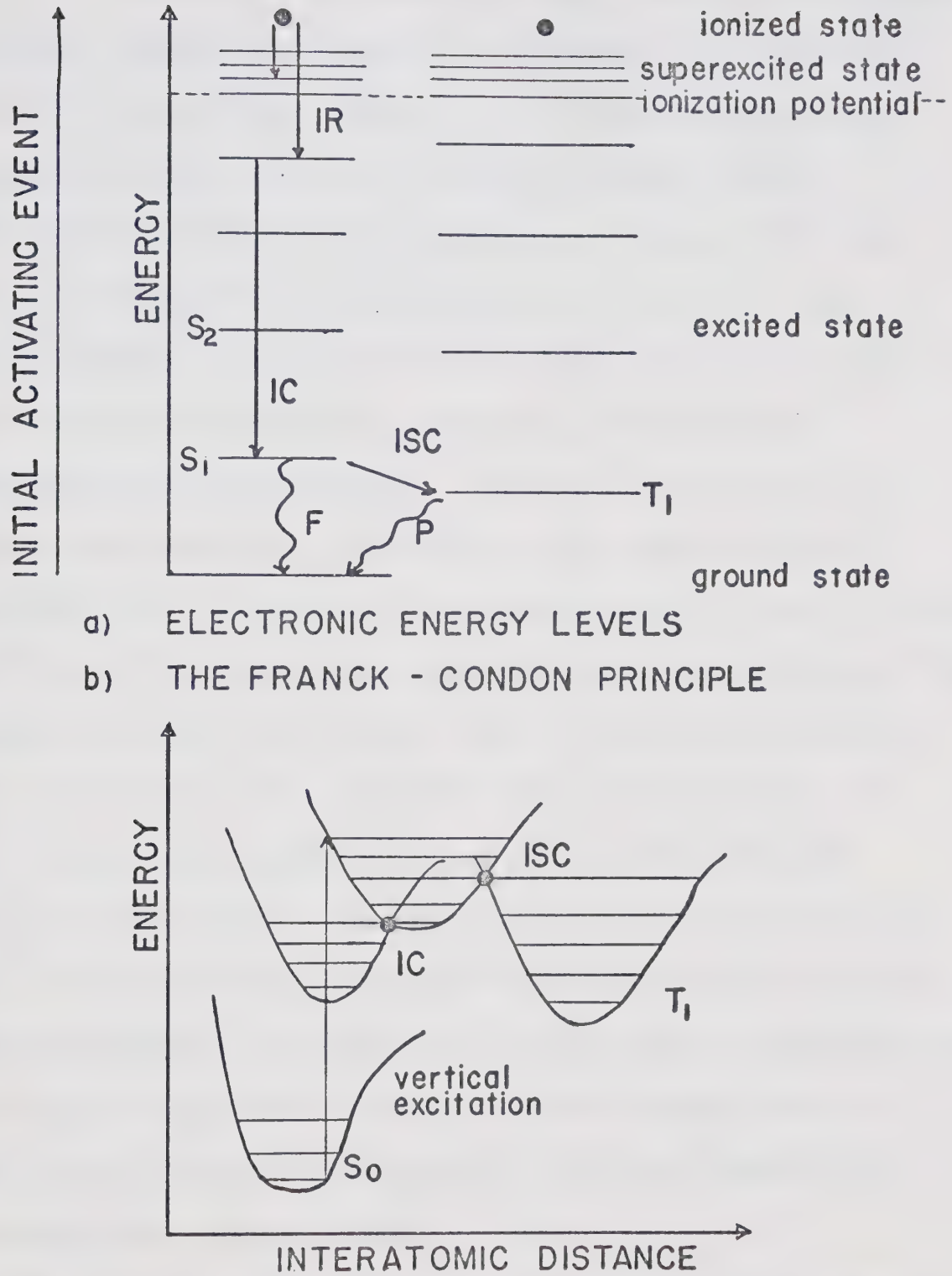


Figure 4. Electronic activation and deactiviation schemes. a) Electronic energy levels. b) Illustration of vibrational energy levels and the Franck-Condon Principle. IR = Ion recombination; IC = Internal conversion; ISC = Intersystem crossing; F = Fluorescence; P = Phosphorescence; S = Singlet states; T = Triplet states.

In the physico-chemical stage, the unstable primary products rapidly deactivate by a variety of uni- and multi-molecular mechanisms. Generally, the fastest modes of de-activation can be expected to dominate (Boag 1967). Electronic processes (ionization) take place in 10^{-16} sec. Vibrational processes take place in 10^{-13} to 10^{-12} sec. These include internal conversion, which leaves the molecule in the first excited state (figure 4), and molecular dissociation (fragmentation). Rotational processes, e.g. dipole re-orientation (solvation), and thermalization of sub-excitation (less than 1 ev) electrons, both occur in about 10^{-11} sec. In water, these processes lead to formation of the aqueous, or solvated electron ($e\bar{a}q$). Fluorescence occurs after 10^{-8} sec. In a condensed medium, such as water at room temperature, neighbouring molecules influence the deactivation process. Frequent inter-molecular collisions (10^{13}/sec) efficiently dissipate vibrational energy in the excited molecule, and encourage recombination of fragments in the molecule undergoing radical or ionic dissociation. It follows that bi-molecular reactions are more important in condensed than gaseous media.

1.1.3 Radiation Chemistry

Molecular species present at the beginning of the

chemical stage are highly reactive. An excited molecule has a lower ionization potential and a higher electron affinity than the ground state (Dainton 1969). The free radical, a molecular fragment with an unpaired electron, is chemically reactive due to the unpaired electron. Because of the chemical instability of the species, reactions are fast and many are diffusion-controlled. Radiation chemical reactions are complete in much less than a second. Some important classes of reactions involving excited molecules, ions, and free radicals are found in table I. It should be pointed out that molecular peroxides formed in reaction C-5 will react slowly, and therefore are able to generate delayed effects (Bacq and Alexander 1961, Spinks and Woods 1964).

1.1.4 Radiation Chemistry of Water

In radiation chemistry and biology it is useful to divide the radiation effect on a molecule into that resulting from direct energy transfer from energetic electrons (direct action), and that mediated by another molecule, typically the solvent molecule (indirect action). The relative contribution of indirect action varies from near 100% in the case of dilute enzyme solutions, to 0% in the case of the irradiation of pure, dry samples. Since the extent of indirect action depends on the yield of reactive species

TABLE I (from Spinks and Woods 1964)

Radiation chemical reactions

A Reactions of Excited Molecules

1. Inter-molecular energy transfer
   $A^* + B \longrightarrow A + B^*$

2. Radical dissociation
   $A^* \longrightarrow R\cdot + S\cdot$

3. Electron transfer
   $A^* + B \longrightarrow A^+ + B^-$

4. Hydrogen abstraction
   $A^* + RH \longrightarrow \cdot AH + R\cdot$

5. Additive reaction
   $A^* + B \longrightarrow AB$

B Reactions of Ions

1. Neutralization
   a) $A^+ + e^- \longrightarrow A^* \longrightarrow M^* + N$ or $R^* + S\cdot$

   b) $A^+ + B^- \xrightarrow{(M)} A\cdot + B\cdot + M^*$

2. Charge transfer
   $A^+ + B \longrightarrow A + B^+$

3. Negative ion formation (electron capture)
   $A + e^- \longrightarrow A^{*-}$ or $B + C^-$

C Reactions of Free Radicals

1. Rearrangement $AB\cdot \longrightarrow \cdot AB$

2. Dissociation $AB\cdot \longrightarrow A + B\cdot$

3. Addition $R\cdot + C=C \longrightarrow R\text{-}C\text{-}C\cdot$

4. H abstraction $A\cdot + RH \longrightarrow AH + R\cdot$

5. Oxygen addition $R\cdot + O_2 \longrightarrow R\text{-}O\text{-}O\cdot$

6. Combination $R\cdot + S\cdot \longrightarrow RS$

7. Electron transfer $M^{Z+} + R\cdot \longrightarrow M^{(Z+1)+} + R^-$

of water, which is the usual solvent in biological systems, the number of solute molecules inactivated is independent of concentration. This "dilution effect" characterizes indirect action.

The primary event in the radiolysis of water is electronic activation, i.e. ionization, excitation, and superexcitation (figure 4).

$$H_2O \xrightarrow{\sim\sim} \begin{matrix} H_2O^+ + e^- \\ H_2O^* \end{matrix}$$

Reactions in the spur follow, and are complete within 10^{-10} sec with yields given in table II (Thomas 1969).

$$H_2O^+ + H_2O \longrightarrow {\cdot}OH + H_3O^+$$

$$H_2O^* \longrightarrow {\cdot}OH + {\cdot}H \,,\; H_2 + {\cdot}O$$

$$e^- + H_2O \longrightarrow .H + OH^-$$

$$e^- + H_2O^+ \longrightarrow .H + \cdot OH$$

$$e^- + (H_2O)_n \longrightarrow e^-aq$$

$$\cdot OH + \cdot OH \longrightarrow H_2O_2$$

Reactions during the uniform period take place in the bulk of the solution between the radiolytic water products and solute molecules. The OH radical is a strong oxidizing agent, ·H and e^-aq are reducing agents. Important reactions are illustrated in table III.

TABLE II

Product yield of spur reactions in neutral water, for low LET radiation (G values)

Molecular Products		Radical Products		
H_2	H_2O_2	OH	H	e^-_{aq}
0.45	0.75	2.6 ± 0.3	0.6	2.6 ± 0.3

TABLE III

Reactions of ·OH, ·H, and e^-aq

---

1. Electron transfer

   a) Oxidative $M^{Z+} + \cdot OH \longrightarrow M^{(Z+1)+} + OH^-$

   b) Reductive $M^{(Z+1)+} + \cdot H \longrightarrow M^{Z+} + H^+$

2. Hydrogen abstraction

$$RH + \cdot OH \longrightarrow R\cdot + H_2O$$

$$+ \cdot H \longrightarrow R\cdot + H_2$$

3. Addition

$$R_2C{=}CR_2 + \cdot OH \longrightarrow R_2OHC - \dot{C}R_2$$

$$+ \cdot H \longrightarrow R_2HC - \dot{C}R_2$$

4. Reactions of e^-aq

   a) Reactions with oxidizing agents (diffusion controlled).

$$e^-aq + O_2 \longrightarrow O_2^-$$

$$H_2O_2 \longrightarrow \cdot OH + OH^-$$

$$MnO_4^- \longrightarrow MnO_4^{2-}$$

   b) Nucleophilic substitution

$$e^-aq + RX \longrightarrow R\cdot + X^-$$

1.1.5 Radiation Protection and Sensitization

The effect of radiation on molecules or organisms may be modified by altering their physical or chemical environment. Radiation protection and sensitization describe those processes which reduce or enhance the expression of ultimate biological damage. These can be classified as fast (less than 10^{-3} sec) radiation chemical processes, and, in the case of cells or organisms in which post-irradiation metabolism is important, slow (greater than 1 sec) biochemical processes (Adams 1970).

Two important radiation chemical protective mechanisms are radical scavenging and hydrogen transfer (Sanner and Pihl 1970). Radical scavengers are compounds which react quickly with radicals, thereby inactivating them and eliminating the associated indirect effect. Sulfhydryl compounds, alcohols and amines are effective OH scavengers. The typical reaction, for a sulfhydyl scavenger is:

$$\cdot OH + RSH \longrightarrow H_2O + RS\cdot$$

$$2RS\cdot \longrightarrow RS\text{ —— }SR$$

Hydrogen transfer occurs by reaction between the protector, typically a sulfhydryl compound, and a molecular radical formed by direct or indirect action.

$$M\cdot + RSH \longrightarrow MH + RS\cdot$$

This repair mechanism may compete with an oxidative fixation step, in which molecular damage is made permanent (Adams 1970).

$$M + O_2 \longrightarrow M\text{-}O\text{-}O\cdot$$

Strongly oxidizing compounds are thought to sensitize both direct and indirect action by electron transfer mechanisms (Adams 1970, Adams et al. 1972). The sensitization due to these compounds, such as dicarbonyls, quinones, and ketones, is anoxic, i.e. it does not add to that produced by oxygen, itself a potent radiosensitizer. Tallentire and Jacobs (1972) have shown a correlation between the extent of radiosentization in anoxic bacterial spores and the electron - affinity of the sensitizer. Other conceivable sensitization mechanisms are 1) removal of an intracellular radioprotector, 2) interference with repair processes, and 3) the radiation-induced production of cytotoxins (Adams and Cooke 1969).

Alteration of various physical parameters may modify the radiation response. The oxygen effect referred to above may be eliminated by anoxic irradiation. Indirect action can be diminished or removed by lowering the temperature to immobilize free radicals (Ebert 1970), and by decreasing the water content of the irradiated sample (Powers and Tallentire 1968). The spatial distribution

of primary radiolytic products of water, and therefore the extent of indirect action depends on the radiation quality (LET) and intensity (dose rate).

1.2 EFFECTS OF RADIATION ON BIOLOGICAL MACROMOLECULES

1.2.1 Biological Macromolecules

Biological macromolecules comprise most of the organic substance of the living cell. These include polysaccharides, lipids, proteins, ribonucleic acids (RNA) and deoxyribonucleic acids (DNA), as well as various conjugated proteins defined by their organic prosthetic group (mucoproteins, lipoproteins, and nucleoproteins). Proteins, and in particular enzymes which have catalytic and metabolic regulatory functions, and DNA, which store and transmit the genetic information that determines the characteristics of the cell, are the macromolecules of obvious biological significance. Accordingly, these are the molecules whose radiation chemistry and biology have been studied as sites of intermediate radiation damage en route to cell death.

1.2.2 Proteins and Enzymes

Proteins are amino-acid polymers of molecular weight 5,000 to more than 10^6 daltons. Enzymatic activity depends on a highly specific molecular structure, determined

by covalent bonds, hydrogen bonds, van der Waals interactions, and salt linkages. The radiation response of proteins and enzymes is treated here by considering first the radiosensitivity of amino-acids and simple peptides and then the radiosensitivity of active enzymes and the mechanisms for radiation-induced loss of enzyme activity.

The amino acid, in solution at neutral pH, exists in the zwitterionic form, $NH_3^+RCHCOO^-$, where R is the side chain. The main products in the radiolysis of simple (aliphatic hydrocarbon side chains) amino acids in oxygenated aqueous solution are ammonia, carbonyls (α-keto acids and aldehydes) and hydrogen peroxide, formed by $\cdot OH$ attack at the $^{\alpha}$-carbon. Preferential $\cdot OH$ attack at aromatic, amino or sulfur containing side chains will decrease the ammonia yield (Garrison 1964). The various amino acids are classified in table IV according to their reactivities with the major water radicals $\cdot OH$ and e^-_{aq}, as determined by bimolecular rate constants (Braams 1966, Anbar and Neta 1967, Okada 1970). As a rule the aromatic and sulfur-containing amino-acids are most reactive.

Irradiation of simple peptides and biologically active oligo-peptides in oxygenated aqueous solution yield amino-acid degradation products, as well as amides and carbonyls resulting from peptide bond cleavage (Garrison 1964,

TABLE IV

Reactivities of amino-acids with •OH and e^-_{aq}

Class	Bimolecular rate constant M^{-1} sec^{-1}	e^-_{aq}	•OH
High reactivity	10^{10}	Sulfur containing CyS-SCy, CySH	. CyS-SCy
			Aromatic aa
			. Met
			. CySH, His
			. Arg
	10^9	. His	. Leu
			. Ile
			. Val
		. Aromatic aa	. Lys
Moderate reactivity			. Ala
	10^8	. Asp-NH_2, Arg	. Ser, Thr
			. Glu
		. Aliphatic aa	. Gly
Low reactivity	10^7	. Hydroxyamino aa	

Garrison et al. 1968, Kopoldova 1968).

The radiation response of proteins and especially biologically active enzymes is complicated by their secondary and tertiary structures. Hydrogen bonding between amino acids, which determines secondary structure (peptide chain configuration) may influence the radiation chemistry around the peptide bond (Garrison and Weeks 1962). Tertiary structure (conformation or folding of the poly-peptide chain) influences the radiosensitivity of an amino-acid residue by determining its ionic environment, its neighbour amino-acids, its relation to the active site, and its accessibility to radical attack (Okada 1970). Thus Braams and Ebert (1968) attribute the 4-fold increase in the reactivity of RNase to e^-_{aq} upon unfolding at elevated temperatures mainly to exposure of hidden disulfide bridges.

The radiation response of most proteins is an exponential inactivation with dose. That dose at which 37% (1/e) of the molecules retain their activity is referred to as the D_{37}. Enzymes irradiated in the dry state have D_{37}'s of about 10 Mrad. Some changes which accompany inactivation after irradiation of dry ribonuclease and lysozyme, following subsequent dissolving in water, are an increase in viscosity, appearance of degraded products, and loss of tightly-bound amide hydrogen atoms. There is no appreciable

amino-acid degradation. These data suggest that the primary cause of inactivation by direct action is conformational change sufficient to destroy the structure of the active site (Okada 1970, Rosen 1971). Enzymes irradiated in dilute aqueous solution are subject principally to the indirect action of water radicals. In these cases, there is detectable amino-acid degradation, especially those reactive residues with sulfur-containing and aromatic side chains, and inactivation may be due to alteration of single amino-acid residues near the enzyme active site (Okada 1970). Enzymes which contain inorganic prosthetic groups may be inactivated by damage to the non-protein moiety. For example, heme enzymes are inactivated by radical action on the ferroprotoporphyrin centre. Lactoperoxidase inactivation was found to correlate with decrease in optical absorption, i.e. destruction of the iron centre (Hamilton et al. 1957), and the addition of the heme-complexing agents cyanide and hydrogen peroxide protected catalase, presumably by shielding the prosthetic group (Sutton 1956).

Because of the complexity of enzyme structure and function, its mechanism of inactivation by radiation is poorly understood. Luse (1964) has presented several modes by which the active site(s) may ultimately be rendered inactive. Indirect action may alter amino acid residues

on the enzyme surface, and form -C or -S radicals by H abstraction. Direct action may cause structural modification due to rupture of weak covalent or polar bonds following intramolecular charge or energy transfer.

1.2.3 Nucleic Acids

Deoxyribonucleic acid (DNA) is a double chain of nucleotides joined by phosphodiester linkages (figure 5). This molecule is highly sensitive to radiation for two reasons. First, it exists as a long chain (molecular weight varies from ca. 10^6 daltons in bacteriophage to over 10^9 daltons in mammalian cells), which presents a large surface area to radical attack. Secondly, because of its specific structure, and the nature of its function, i.e. transmitting regulatory and genetic information, small radiation damage may be amplified in subsequent biological expression (Latarjet 1972). For these reasons, (especially the latter), DNA is considered as the likely molecular "target" in radiation damage to a cell (Chadwick and Leenhouts 1973, Okada 1970).

We will examine the response of DNA to radiation first by considering the sensitivity of its constituents.

The radiation chemistry of nucleotides is determined mainly by that of the purine and pyrimidine

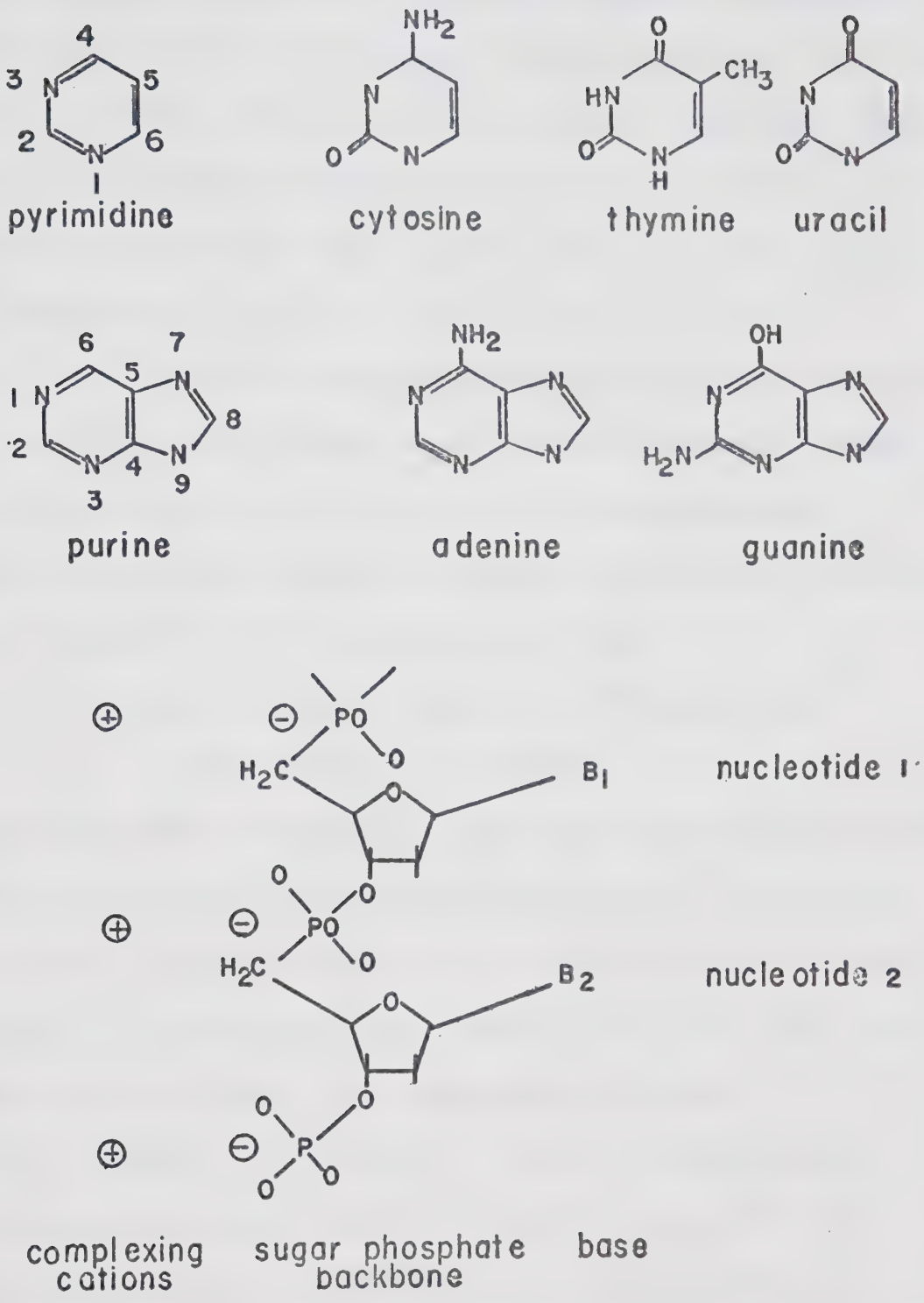


Figure 5. Structure of DNA and its constituent bases.

bases (figure 5). All but 20% of OH radicals produced in aqueous solutions of pyrimidine nucleotides react with the bases, and 75% react with bases in purine nucleotides (Scholes 1968). The pyrimidine bases are very reactive with water radicals, and purines only slightly less so. Rate constants for reaction with $\cdot OH \simeq 4 \times 10^9\ M^{-1}\ sec^{-1}$, and with $e^-aq \simeq 10^{10}\ M^{-1}\ sec^{-1}$ (Scholes 1968, Greenstock et al. 1968).

The major product in oxygenated neutral aqueous solutions of pyrimidines is a hydroxy-hydroperoxide, resulting from $\cdot OH$ addition at the 5, 6 double bond (Scholes 1968), according to the scheme shown below:

$$pyr + \cdot OH \rightarrow pyr(OH) \xrightarrow{O_2} pyr(OH)O_2$$

$$\rightarrow pyr(OH)O_2H + \text{products}$$

These compounds are unstable, especially the hydroperoxides of uracil and cytosine, and decompose readily (Schwiebert and Daniels 1971). As expected by the high reaction rate constants, pyrimidines react quantitatively with $\cdot OH$, and $G(-pyr) = 2.5 \simeq G(\cdot OH)$. In deaerated solutions, $G(-pyr) < G(\cdot OH) + G(e^-aq) + G(\cdot H)$, indicating back reactions of radical adducts. Cytosine undergoes $\cdot OH$ or e^-aq attack at its 3, 4 N — C double bond, leading to deamination and a significant yield of uracil (Scholes 1968).

Purines have approximately half the sensitivity of pyrimidines. Since their reactivity is not significantly

less from that of pyrimidines, this fact suggests that a sort of chemical restitution occurs (Latarjet 1972). Major reactions in oxygenated solutions involve ·OH addition to the central 4-5 bond, and in oxygen free solutions, ·OH and ·H addition to the 7-8 bond, leading to rupture of the imidizole ring (von Hemmen and Bleichrodt 1971).

The reactivity of nucleotides, expected to be ca.25% greater than that of the pure bases because of the contribution of the deoxyribose moiety, in fact is unchanged for reaction with ·OH and decreased by half for reaction with e^-_{aq} (Scholes 1968, Greenstock et al. 1968). The base is therefore deactivated, perhaps by influence of the ionized phosphate group. The reactivity per base of model oligo- and poly-nucleotides is less than that of free bases, and this decrease is explained by a decrease in collision frequency per base (Shragge et al. 1971). Similar results in double-stranded polynucleotides indicate that bases bound in the hydrogen bonded network of the double strand are afforded further protection by this structure.

Irradiation of dilute aqueous solution of biologically active DNA yields base destruction and base or nucleotide liberation. These effects result from radical (mainly ·OH) attack at the base and the sugar respectively with the expected relative frequency of 4 : 1 (Ullrich and

Hagen 1971, Blok and Verhey 1968, Scholes 1968). Single strand breaks of the sugar-phosphate chain occur with radical attack on the sugar moiety. The G-value of single strand breaks in a 0.1% solution of calf thymus (double stranded) DNA is 0.4, compared to 1.7 for base destruction (Collyns et al. 1965).

Direct action on dry or frozen DNA samples causes primarily single and double strand breaks. These may be directly due to single or multiple ionizations at the phosphodiester bond, or by localization of excitation energy at energy sinks in the nucleotide chain. Gregoli and Bertinchamps (1972) have shown a complete spin (electron) transfer to pyrimidines (mainly thymine) in ESR studies on freeze dried DNA. Fielden et al. (1971) have shown by luminescence kinetics that excitation energy will migrate over 100 base pairs and localize on bromodeoxyuridine, a thymine analog whose incorporation in DNA increases strand break yield (Sawada and Okada 1972).

The biological implications of radiation-induced DNA lesions are manifest: base destruction leads to mutagenesis and strand breaks lead to chromosomal aberrations. However, before applying these physico-chemical results to phenomena at the cellular level, we must consider the biology of the cell.

1.2.4 Radiation Chemistry and The Cellular Environment

The results of *in vitro* radiation studies on biological macromolecules can be extended to explain cellular responses only if caution is exercised. The intracellular distribution of molecules is complex. The cellular environment is heterogeneous with respect to physical factors such as pH, ionic concentration, and O_2 tension. Furthermore, cellular molecules can be found in various chemical environments such as aqueous or non-aqueous suspensions, molecular aggregates, and complexes with membranes or with other molecules. All of these factors are important in determining molecular radiosensitivity. In particular, the influence of molecular complexes on radiosensitivity is considered here.

Enzyme-substrate complexes have been mentioned above in reference to hemoprotein-substrate or inhibitor complexes. The complexing agent in these cases protects the enzyme in aqueous solutions, probably by shielding the binding site from radical attack. DNA in eukaryotic cells exists in permanent association with basic proteins. Luminescence studies on direct action in frozen DNA-histone complexes show a complete transfer of excitation energy to DNA, representing 40% of the total energy deposited in the protein (Lillicrap and Fielden 1972). This supports

earlier evidence of greater DNA radical yield due to direct action on nucleo-protein than DNA alone. (Alexander et al. 1961, referred to in Okada 1970). On the other hand, irradiation of aqueous solutions of nucleoprotein and pure DNA indicate significant protection by the protein component (Bacq and Alexander 1961) although there does occur some secondary delayed degradation in the DNA, probably due to the action of stable radicals formed in the protein sheath (Latarjet 1972, de Jong et al. 1972). On the whole, therefore, it can be expected that the protein will act as a protector in the cellular environment.

The radiosensitivities of biologically sensitive macromolecules are such that radiation doses lethal to a mammalian cell (100 rads for L5178Y (figure 9)) will cause very slight damage. This, coupled with the nature of radiation-induced reproductive death in mammalian cells, whereby a cell may metabolize through several life cycles before dying (Watanabe and Okada 1966), points to a latent period during which the initial radiation damage inflicted on cellular molecules is amplified. The amplification process may involve faulty or inadequate macromolecular synthesis resulting from template DNA damage (Hopwood and Tolmach 1971). Other possibilities include breakdown of extensive interdependent multi-enzyme metabolic systems,

and structural damage to cellular or sub-cellular membranes (Okada 1970).

CHAPTER 2

RADIATION BIOLOGY OF MAMMALIAN CELLS

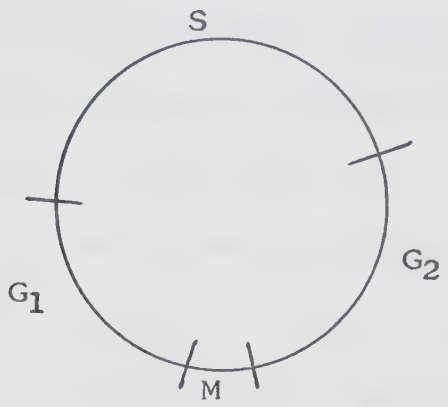
2.1 CELLS IN CULTURE

Cells which constitute any reasonably complex organism represent a widely heterogeneous population. The mammalian organism, for example, is comprised of various types of cell populations which can be classified according to their kinetics. These are defined by the input of cells from other populations, and the loss of cells to other populations or through death. Thus, the adult mammal has stationary and dynamic steady - state populations, decaying populations and growing populations. Each cell population has its own biological characteristics. We shall consider that of a growing population, the closed dividing cell system, and in particular the *in vitro* model of cells of established lines in tissue culture.

Biological and radiobiological studies on cultured cells are useful for several reasons. Tissue culture is less costly and more convenient for study of cellular processes than *in vivo* systems. Also, tissue culture represents a good model for some tumors. Cultured cells have undergone similar dedifferentiation of function, metabolic changes, and aneuploid transformations as have malignant cells.

Cells in a proliferating culture follow a so-called cell cycle, each point in which can be identified by

some characteristic biochemical or physiological event. In practice the cell cycle is divided into four stages: G1, during which RNA and protein synthesis begins; S, the total time during which DNA undergoes normal replication; G2, during which macromolecular synthesis and energy accumulation are completed; and M (mitosis), when the cell divides.



The cell-cycle parameters in a growing, asynchronous culture are reasonably uniform, and can be determined by a variety of experimental and analytical methods (Okada 1967). By taking advantage of the biochemical and physiological properties of cells in the cell-cycle, it is possible to select cells at one stage, and thus form a homogeneous culture of synchronous cells. That synchrony decays appreciably within one cycle is evidence that no culture can be truly homogeneous.

The growth of cells in culture can be described in four phases. Upon inoculation in new culture, there is no increase in multiplicity as the cells adapt and increase in

size (lag phase). After adaptation of the cells, the culture enters log phase during which the cell number increases exponentially with a doubling time usually slightly longer than the mean cell cycle time. The difference is due to a small proportion of non-viable (non-cycling or dying) cells. The total metabolic activity of the culture is most vigorous during early log phase. Later, because of depletion of medium nutrients, accumulation of growth inhibitory factors (Froese 1971), cell density inhibition, and other factors, cell growth slows and the number of non-viable cells increases, as the culture enters stationary phase. Finally, cell number decreases in the culture's dying phase. Sub-culturing or diluting in fresh medium is generally done from late log phase to minimize the lag phase. In suspension culture, lag phase can be virtually eliminated by judicious dilution.

Radiobiological phenomena in cultured cells will now be discussed. Wherever possible, reference will be made to results with mouse leukemic lymphoblasts, line L5178Y, the system which was used in this study.

2.2 RESPONSE OF CULTURED MAMMALIAN CELLS TO RADIATION

2.2.1 Survival Curves

It was mentioned earlier that established cell lines *in vitro* bear biological resemblance to some tumors. Much radiobiological study of cultured cells has been pursued in the hope of gaining understanding of the response of tumors to radiotherapy. Therefore, considerable work has been done in the moderate dose range (around 10^3 rads) on the radiotherapeutically important response, viz. reproductive death. This is defined as suppression of the unlimited proliferative ability of a cell, and is not necessarily accompanied by lysis or metabolic death (Elkind and Whitmore 1967). According to this criterion, survival following irradiation is assayed by counting the number of cells in the treated population which retain their proliferative ability. This is done directly by placing a known inoculum of treated cells on an immobilizing substrate (a plastic or glass surface, or agar gel), incubating, and counting the macroscopic cell clusters (colonies), one of which results from each viable, or proliferating cell. Since cells do not grow well at low cell densities, conditioning factors are generally added to the assay mixture. Such factors are x-ray sterilized cells (feeder cells), and conditioned medium. Under these conditions,

plating efficiencies (ratio of colonies to initial cell number) of control cultures are routinely 50 to 100%. This modification of the common microbiological assay was developed for mammalian cells by Puck and Marcus (1956), and represented one of the more important advances in quantitative cellular radiation biology. Another technique used to determine the viability of irradiated cells, that of growth extrapolation, is described in Chapter 4.

Survival curves of most cultured mammalian cells irradiated in log phase are sigmoidal, with a shoulder at low dose, indicating accumulating reparable damage, and exponentially decreasing survival at higher doses. For these systems the survival-dose relation is described by the equation,

$$S = 1 - (1 - \exp(-D/D_o))^n$$

where S is the fraction of cells surviving irradiation of dose D. The parameters D_o and n represent respectively the dose corresponding to 1/e (0.37) survival on the exponential portion of the curve, and the survival intercept at zero dose of the extrapolated exponential portion. They also have theoretical significance in target theory of radiation action (see Lea 1956), but this will not be discussed here. The survival curve presented in figure 9 is typical of cultured mammalian cells, and yields

D_o = 100 rads and n = 2.0.

The phenomenon of reproductive death will be mentioned again. Meanwhile some of the biological effects preceding cell death are examined.

2.2.2 Biochemical Effects of Radiation: Macromolecular Synthesis

Normal cell progression and division depends on synthesis of a normal macro-molecular complement. Radiation effects on biosynthetic patterns are antecedent to, and perhaps directly responsible for disturbances of cell progession.

The rate of DNA synthesis during S stage is depressed soon after irradiation, and the degree of depression is dose-dependent. The duration of S stage is increased, so that the total amount of DNA synthesized in irradiated cells is comparable to, or greater than that in control cells (Weiss 1971). However, total DNA synthesis is deficient in the second post-irradiation generation. Hopwood and Tolmach (1971) reported that the fraction of DNA synthesized is 67% in the average daughter cell, and only 30% in those daughter cells which did not subsequently divide after the second generation. Structural damage to the DNA molecule was suggested as a cause of deficient DNA

synthesis which seems specifically to precede cell reproductive death.

DNA synthesis has been detected in non-S stage cells exposed to moderate doses. This "unscheduled replication", associated with repair, is complete 1 to 2 hours after irradiation, and its activity is dose-dependent. Brent and Wheatley (1971) noted that most of the repaired DNA underwent subsequent replication in S-stage, indicating retention of functional integrity. Rejoining of DNA single-strand scissions following heavy irradiation of L5178Y cells occurred for 1 hour after irradiation (Sawada and Okada 1970), and involved about 10 bases per break (Fox and Fox 1973).

RNA synthesis is somewhat less radiosensitive. Inhibition of the rate of RNA synthesis was shown in HeLa cells 1 to 2 hours after irradiation (Kim et al. 1970, Bases et al. 1970); however, Bacchetti and Sinclair (1971) noticed a slight stimulation in Chinese hamster cells. The inhibition was shown to be due to depressed ribosomal RNA (r-RNA) synthesis, with a concommitant decrease in new ribosomal particles, while no effect on messenger RNA (m-RNA) was seen (Bases et al. 1970). This observation was confirmed by Meltz and Okada (1971), who showed that an increased proportion of RNA synthesized after irradiation of L5178Y cells was DNA-hybridizable, i.e. m-RNA. The

hybridization of "irradiated" m-RNA was indistinguishable from that in control cells, indicating that irradiation caused no gross change in base sequence of the m-RNA, nor, therefore, in the transcribed DNA.

Relying as it does on an integral DNA template and m-RNA molecule, and properly functioning ribosomal machinery, protein synthesis is expected to be at least as radiosensitive. Consistant with the observations made earlier regarding the radiosensitivities of the DNA template and m-RNA synthetic process, as well as the report of Tobey et al. (1970) that polyribosomes in the irradiated cells are able to synthesize functional virus proteins, overall protein synthesis following irradiation is indeed unaffected, or slightly stimulated (Bacchetti and Sinclair 1970). It must be remembered that, in view of the diversity and specificity of protein and enzyme function, total synthesis is hardly a sensitive test of radiation damage. Deficient synthesis of specific proteins required for mitosis has been suggested to be responsible for radiation-induced mitotic delay (Doida and Okada 1969).

2.2.3 Physiological Effects of Radiation

Radiation effects on cellular physiology are, as a rule, less complex and easier to detect than chemical and

biochemical changes, and are useful indicators of radiation damage to cells.

Cell progression is altered by radiation at two sites in the cell cycle. Prolongation of S stage has been discussed. More dramatic is a complete, temporary block in late G2 which contributes, with S prolongation, to mitotic (or division) delay. Mitotic delay can be detected in asynchronous cultures by a rapid drop in mitotic index (the fraction of cells in mitosis), or by a halt in cell multiplication immediately after irradiation (the latter effect is, strictly, division delay). It is dose-dependent and stage-dependent. The site of the block in G2 coincides with those due to puromycin (Doida and Okada 1969) and cycloheximide (Walters and Petersen 1968), both of which stop protein synthesis at the level of translation. In addition, recovery from the G2 block is prevented in the presence of these agents. These data suggest that mitotic delay due to the G2 block is caused by deficient synthesis of proteins necessary for mitosis. It is interesting to note that cell survival was enhanced by post-irradiation treatment with cycloheximide, (Bacchetti and Sinclair 1971), suggesting that radiation-induced mitotic delay and reproductive death are independent and unconnected processes.

Differential responses to irradiation of cells

at different positions in the cell cycle is known as stage (or age) dependency. Some of these responses and their stage dependencies (most sensitive to most resistant) are reproductive death and chromosome aberrations ($M \simeq G1 > G2 > S$), mitotic delay ($S > G_2 > G_1$), and DNA synthesis (late S > early S) (Sinclair 1968, Okada 1970). The reasons for stage dependency are, for the most part, obscure. Sinclair (1969) reported that Chinese hamster cells are differentially protected through the cell cycle by the sulfhydryl radioprotector, cysteamine, with the net effect of partially levelling the survival dependence on cell stage. This was taken to indicate that at least some of the stage dependency of reproductive death is due to fluctuating levels of intracellular non-protein SH groups through the cell cycle. Based on the similarity of chromosome aberration and survival stage dependencies and on the correlation between the separation of chromatin fibres and survival through the cell cycle, Dewey et al. (1972) suggested that stage-dependent lethality is due to interactions of single lesions in chromatin fibres.

A characteristic radiation-induced disruption of the cell cycle is giant cell formation. This is seen extensively in cultures which have been exposed to moderate to high doses. Giant cells metabolize normally but do not undergo mitosis after the second post-irradiation cycle

(virtually all cells divide at least once), resulting in increased volume, macromolecular complement, and polyploidy. The life cycle of giant cells is thought to be G_1, S, G_2, M^*, G_1... where M^* represents abortive mitosis without division, or mitosis with division followed by fusion (Okada 1970). Giant cells rarely reattain normal mitosis, and are non-viable according to the definition of reproductive death.

Radiation effects on membranes will be discussed later in connection with radiation-induced interphase death.

2.2.4 Cytological Effects of Radiation

Changes in chromosomal morphology have been recognized and studied in cells exposed to ionizing radiation for many years (see e.g. Lea 1956). Corresponding study with cultured mammalian cells is more recent, and will be briefly mentioned here. (See Elkind and Whitmore 1967 for a more extensive review).

According to a current model of chromosome structure, the G_1 cell contains chromosomes, each of which consist of two chromatids. These spread apart at G1/S, replicate during S stage, and separate during mitosis into the daughter cells. Thus, each G_2 chromatid, consisting of

two strands becomes the two chromatids of the subsequent G_1 chromosome.

Chromosomal morphology is visible only during mitosis, therefore chromosomal aberrations in cells irradiated during interphase must be scored in M. The aberrations seen in cultured cells after moderate doses are of two types: those due to chromosome breaks (chromosome-type aberrations), which predominate in G_1 before splitting and replication of the chromatin, and those due to chromatid breaks, occurring mainly in S and G_2. The occurance of chromosomal aberrations is similar in many respects to cell reproductive death. For instance the two responses share the same stage-dependence and LET-dependence (Elkind and Whitmore 1967), both exhibit similar repair of sub-lethal damage (Okada 1970) and potential-lethal damage (Winans et al. 1972). There is, as well, a reasonable correlation between radiosensitivity (D_o) and total chromosomal volume within classes of organisms (Okada 1970). These, and other data suggest that radiation effects on chromosomes and on cell survival are, at least, connected events (Dewey et al. 1972).

2.2.5 Recovery from Radiation Damage

Repair of radiation-damaged DNA was mentioned earlier. In this section we consider recovery at the

cellular level, i.e. that which is manifested by increased cell survival.

Mammalian cells lack an efficient mechanism for repair of lethal damage (Elkind and Whitmore 1967); they certainly are not capable of photo-reactivation which is so important for U V treated bacteria. However, the characteristic sigmoidal survival curve suggests that irradiated mammalian cells are able to accumulate sub-lethal damage, and the work of Elkind, Sutton and associates have shown that this damage is completely reparable (see Elkind's review (1967) and references therein). Repair of sub-lethal damage can be shown by giving a conditioning dose, D_c , to a cell culture, such that the resulting survival $S(D_c)$ is at the junction of the shoulder and exponential portion of the survival curve where the accumulation of sub-lethal damage is maximal (figure 6). This is followed by a second dose D_2 given as a function of time after D_c . The resulting survival increases from $S(D_c + D_2)$ at $t = o$, to $S(D_c) \times S(D_2)$, after 10 hours, where it levels off. (The survival values $S(D_2)$ and $S(D_c + D_2)$ would have indicated respectively repair of both lethal and sub-lethal damage, and no repair at all). A statement equivalent to "repair of sub-lethal damage" is "restoration of the survival curve shoulder". Work with synchronized

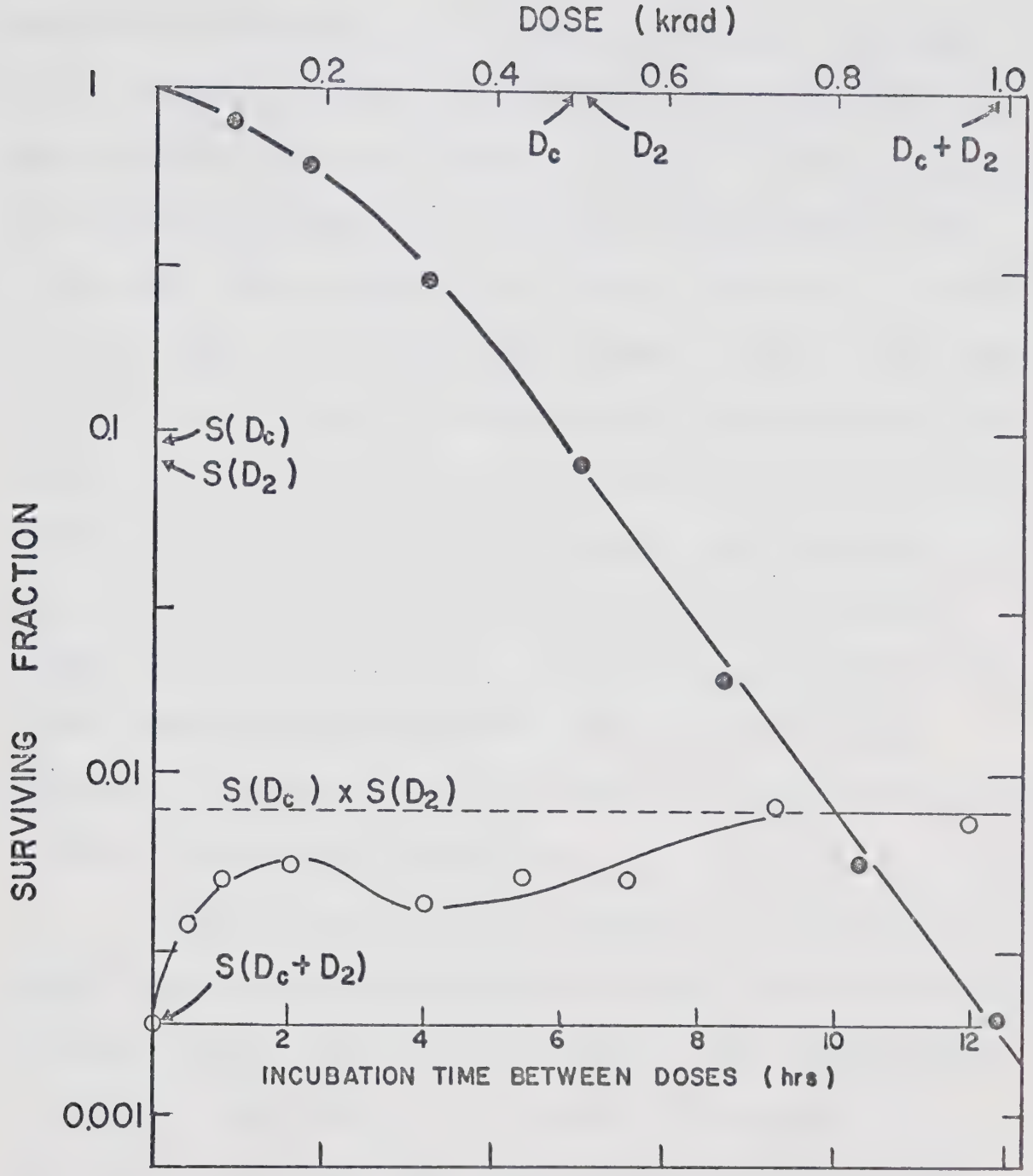


Figure 6. Repair of sub-lethal damage. Survival curve (closed circles) and 2 - dose fractionation curve (open circles) of Chinese hamster cells. (After Elkind and Whitmore 1967).

populations has shown that the observed "repair" response is attributable to a real intracellular, stage-dependent, repair process initiated immediately after the first dose, and superimposed upon this, variations in radiosensitivity as the cells progress around the cycle. Addition of various metabolic inhibitors to the cells between doses indicate that macromolecular synthesis is required for progression but not repair. Low inter-dose incubation temperature has the same effect, leading Elkind (1967) to suggest that the early repair is a passive (non-enzymatic) process. Sub-lethal damage repair is not limited to single inter-dose intervals; indeed, many-dose fractionation schemes are the rule in radiotherapy, and had been long before in vitro studies of sub-lethal damage were carried out.

When cultured cells are exposed to various sub-optimal growth conditions after irradiation, their survival increases (Okada 1970). The dependence on post-irradiation treatment is considered to be a result of competing processes of repair and confirmation of potentially-lethal damage (Phillips and Tolmach 1966). Thus, increased survival with post-irradiation treatment can be due to suppression of the confirmation process and/or enhancement of the repair process. Factors which have this effect are cycloheximide, reduced temperature (Winans et al. 1972), incubation in

nutritionally limited medium (Belli and Shelton 1969), and the presence of some component in conditioned medium (Little 1971). Factors which enhance confirmation of damage and/or suppress repair are inhibitors of DNA synthesis and some other inhibitors of macromolecular synthesis, puromycin and actinomycin D (Okada 1970). Based on studies with actinomycin D, Elkind et al. (1967) concluded that characteristics of sub-lethal and potentially-lethal repair were similar, and suggested that the natures of the two types of damage were different only in degree. Winans et al. (1972) recently proposed that potentially lethal damage resulted from the interaction of single chromatin lesions (considered to be sub-lethal damage).

2.2.6 Mechanisms of Cell Death

Up to now, the radiobiological phenomena considered have been those which occur in a culture of cells undergoing reproductive death. We have seen, in connection with mitotic delay, giant cell formation, and the stage-dependence of reproductive death, that mitosis is a very radiosensitive stage. Indeed, it has been shown that mitosis plays an essential role in the eventual dying process. Watanabe and Okada (1966) followed the course of dying L5178Y cells in an irradiated culture by determining the fraction of cells stainable with a dye which is excluded in healthy cells. For doses up to 10 krad, increases in number of degenerative

cells were immediately preceded by peaks in mitotic activity. Furthermore, when the mitotic peak was delayed by a second dose of radiation, the appearance of dying cells was likewise delayed (Okada 1970). For reasons mentioned earlier, reproductive death is thought to be associated with chromosomal lesions. However, the molecular events responsible for these lesions are uncertain.

Radiation-induced interphase death, which is not associated with cell division, predominates at higher doses for proliferating cells in culture and at moderate doses for non-dividing cells, e.g. lymphocytes and erythrocytes. Interphase death is characterized by cell death several hours after irradiation, indicated by loss of dye-exclusion ability. Biochemical and physiological alterations which precede cell death involve depression of ATP production, disruption of membrane permeability, and disorganization of nuclear structures (Okada 1970). It is likely that these processes are inter-dependent.

Radiation effects on the membrane are of interest because of its role in interphase death. The integrity of the plasma membrane, as well as membranes of the intracellular organelles, such as mitochondria, lysosomes, nucleus, and endoplasmic reticulum, is essential for normal cell function. Similar effects are seen after irradiation

of both isolated sub-cellular fractions and whole cells. The protein moiety is attacked chiefly at sulfur bonds; oxidation of sulfhydryl groups, and disruption of disulfides both occur. These reactions disrupt specific enzyme activity of the membrane, besides rendering the membrane proteins more sensitive to attack by proteolytic enzymes (Myers 1970). Lipid peroxidation occurs during post-irradiation incubation in plasma membranes of intact cells (Myers 1970) and in the endoplasmic reticulum (Wills 1970).

The relationship of membrane damage to the other concurrent phenomena associated with interphase cell death, i.e. depressed oxidation and nuclear phosphorylation, and the appearance of pycnotic (degenerative) nuclei, is not clear.

2.3 CONCLUSION

Having considered first the immediate chemical actions of ionizing radiation on biological material, and secondly the ultimate expression of biological damage, it is clear that the intervening steps, i.e. the initial biochemical lesions and subsequent development of damage, are obscure. One of the reasons for the difficulty in understanding biological effects of radiation is that its action is non-specific, and by implication therefore, it is able to act on sensitive sites and functions in various ways. In the following chapters,

we consider only that indirect action mediated through long-lived species which have an extracellular origin.

CHAPTER 3

THE CULTURE MEDIUM IN CELLULAR RADIATION BIOLOGY

3.1 BIOLOGICAL FUNCTION OF THE CULTURE MEDIUM

Maintenance of mammalian cells in culture requires control of a number of physical and chemical factors in the cellular environment, provided by the culture medium.

The osmotic pressure of the culture medium for optimal cell growth is generally close to that of the original cellular environment, i.e. the body-fluid. All solutes contribute to the osmotic pressure of the medium, however NaCl is mainly responsible for maintaining tonicity.

Most mammalian cells require near-neutral pH (6.6 to 7.8) to survive, and attain optimal growth in a narrow pH range around 7.3.

The buffer most widely used is the bicarbonate - carbon dioxide system. This utilizes the equilibrium between liquid and gas phases, and the pH fluctuates according to the equilibrium. The pH may be stabilized by adding one or several of non-volatile organic buffers (Good et al. 1966, Eagle 1971).

Besides regulating medium tonicity and hydrogen ion concentration, inorganic ions are required for other reasons. For example K^+, Na^+, NH_4^+ Ca^{2+} function to stimulate some enzyme activity (Mahler and Cordes 1966), Mg^{2+} acts as a

cofactor in a number of kinases, and Fe^{2+} is in the nucleus of the heme proteins and enzymes. The bicarbonate ion is found throughout cellular metabolism, and the phosphate ion is central in energy metabolism through high energy phosphate bonds (Paul 1970).

Although not dependent on the medium, sufficient thermal energy must be provided for cellular processes to take place. Metabolism is slowed at sub-optimal temperatures, e.g. L5178Y cell progression is virtually stopped at 28°C (Watanabe and Okada 1967). On the other hand, tolerance to hyper-thermic conditions (over 40°C) is limited. The viable population of Chinese hamster cells *in vitro* is reduced to 1/e when incubated at 43.5°C for 7 minutes (Westra and Dewey 1971).

A very important function of the culture medium is nutritional; i.e. the supply of essential metabolites. Carbohydrate, generally glucose (although some other hexoses are equally effective (Paul 1965), is supplied as an energy source and a carbon source. Energy is obtained in the form of high-energy phosphate bonds (ATP) either from the anaerobic glycolytic pathway, or the aerobic pentose-phosphate pathway and Krebs cycle. Nucleosides and amino-acids are synthesized from intermediates in the aerobic metabolic pathway. Oxygen and carbon dioxide must be present in the medium for aerobic

metabolic activity. The medium must contain amino-acids which the cell cannot synthesize, (the so-called essential amino-acids), and certain B vitamins are required as co-factors in essential enzymatic reactions (Paul 1970).

Few cell lines are known which grow in a chemically defined medium containing only the essential metabolites mentioned above. Most cell lines have unspecified protein requirements which are satisfied by a serum supplement (usually about 10% v/v). In addition, protein factors in serum provide a chemical and physical function in facilitating attachment to glass in monolayer cultures, and in rendering cells in suspension less fragile by increasing the viscosity of the medium.

In discussing tissue-culture nutrition, it should be borne in mind that nutritional requirements are not rigid. Some metabolite requirements are cell-density dependent, and cell lines can adapt over a period of time to different regimens (Eagle and Levintow 1965). Finally, the physical condition, e.g. gas tension and pH, and chemical composition of the culture medium (Austin et al. 1971) are altered by metabolizing cells.

3.2 RADIATION EFFECTS ON THE MEDIUM

3.2.1 Radiation Chemistry

a) Whole Medium

The most complex culture medium is a reasonably dilute aqueous solution, containing $\simeq 10^{-1}$ M Cl^-, $\simeq 10^{-2}$ M glucose and HCO_3^-, and $\lesssim 10^{-3}$ M amino acids (appendix I). For this reason, it is expected that direct action is negligible. Since the medium is in equilibrium with air, the reducing radicals H and e^-_{aq} should be rapidly scavenged by O_2. The radiation chemistry of an aerated culture medium, then, is due mainly to indirect action of the oxidizing water radicals $\cdot OH$, $\cdot O_2^-$ and $\cdot HO_2$. The hydroperoxy radicals $\cdot O_2^-$ and $\cdot HO_2$ are not strong oxidizing agents and will react mainly with other radicals (Spinks and Wood 1964).

The solutes in defined Fischer's medium (appendix I) complete for reaction with $\cdot OH$ according to the product of their concentrations and reactivities (Table IV) (Hayon 1968). Accordingly glucose (40%), methionine (20%), and tyrosine, glutamine, phenylalanine and histidine (ca. 10% each) of the defined small molecule complement can be expected to react with $\cdot OH$ in the proportions indicated. In whole medium, the serum macro-molecules concentration is less than 1%. At this concentration, and using the reactivity for serum albumin of 2×10^{10} M^{-1} sec^{-1} (Anbar and Neta 1967), the

undefined macromolecular complement of whole medium will scavenge 10-20% of ·OH radicals.

The radiation chemistry of such a mixture is impossibly complex. However, studies have been done on the radiolysis of single component solutions. Phillips et al. (1958) indentified the major products in the radiolysis of neutral, oxygenated aqueous solutions of glucose as glyoxal (G = 1.8), glucuronic and gluconic acids (G = 0.9 and 0.4), and hydrogen peroxide (G = 2.0). Radiolysis of methionine yields ammonia and a carbonyl due to ·OH attack at the carbon, and also suffers attack at the sulfur moiety (Garrison 1968). The aromatic amino-acids are probably attacked in the side chain ring (Garrison 1968). The water radiolytic yield of hydrogen peroxide should not be significantly decreased by reactive solutes at the concentrations present (Kupperman 1967). Indeed, the total yield may be enhanced through secondary reactions (Phillips et al. quoted above, and Frey and Pollard 1966).

Although products of medium radiolysis are physico-chemically stable, some are chemically labile and may undergo secondary reactions for some time after irradiation. Peroxides, in particular, can react by nucleophilic substitution or addition (Bunton 1962).

$$RO_2H + -C-X \longrightarrow RO_2C- + HX$$

$$RO_2H + C = X \longrightarrow RO_2C-X + H^+$$

possible subsequent hydrolysis

They can also act as electrophiles in the presence of strong ionizable nucleophiles, e.g. halides (Edwards 1962).

$$R'OOR + N^- \longrightarrow R'O^- + RON$$

Schubert et al. (1967) reported biological evidence suggesting formation of toxic hydroxyalkyl peroxides by nucleophilic addition of hydrogen peroxide to aldehydes and ketones formed in the radiolysis of aqueous sucrose solutions.

Hydrogen peroxide may be enzymatically decomposed after irradiation. Perlmann and Lipmann (1945) reported 15-20 μg catalase/100 ml human serum, and Lieberman and Ove (1958) found intracellular catalase in cells in culture. Catalase reacts rapidly with hydrogen peroxide to form water and oxygen.

b) Saline Solution

The radiation chemistry of phosphate-buffered saline should be determined by reactions between ·OH and its constituent anions, Cl^- (0.14M), HPO_4^{2-} (0.013M), and $H_2PO_4^-$ (0.002M) (Appendix I). The reactivity of the chloride ion with ·OH is pH dependent, first order with respect to H^+ (Ward and Myers 1965). However, Anbar and Thomas (1964) identified an intermediate species formed in pulse radiolysis

of neutral, aqueous chloride solutions as the radical anion Cl_2^-. The authors propose that Cl· is formed in the spur, an acidic micro-environment, and combines rapidly with Cl^-, for Cl^- concentrations greater than about 0.1M.

$$H_3O^+ + \cdot OH + Cl^- \longrightarrow Cl\cdot + 2H_2O$$

$$\cdot Cl + Cl^- \longrightarrow Cl^-_2$$

The yield of Cl^-_2 was unaffected by the presence of 0.5 M phosphate. Langmuir and Hayon (1967) studied decay schemes of Cl^-_2 and obtained rate constants for its disappearance,

$$2Cl_2^- \longrightarrow 2Cl^- + Cl_2, \quad 2k \approx 10^{10}\ M^{-1}sec^{-1}$$

and reactions with other solutes, notably hydrogen peroxide, $k(Cl^-_2 + H_2O_2) = 7 \times 10^7\ M^{-1}sec^{-1}$. Products of chloride ion radiation chemistry are likely chemically labile, and can be expected to react readily following radiolysis.

3.2.2 Biological Effects of Irradiated Medium

a) Introduction and History

Indirect action in radiation biology can, in practice, be separated into rapid and slow components. The former, mediated primarily through radicals and unstable intermediates in the medium, is complete in less than 1 sec. The latter component is due to reaction with stable radiation-induced products formed in the medium, and it will act as

long as the products are present. In the case of cellular radiation effects, the rapid indirect action originates in the cell water (Bacq and Alexander 1961). The slow indirect action originates mainly outside the cell because of the larger volume of extracellular water. Therefore, indirect action due to long-lived radiation-produced species may be studied free of contamination from other radiation effects by exposing unirradiated cells to irradiated medium.

Radiation workers have long been aware of biological effects of irradiated medium. Taylor et al. (1933) showed that exposure to irradiated yeast extract medium or medium made up with irradiated water was toxic to the protozoon Colpidium campylum. Blank and Kersten (1935) attributed growth inhibition of B. subtilus growing on irradiated medium to the agar component. Evans (1947) concluded that the agent which reduced the survival time of sea urchin sperm in irradiated sea water was hydrogen peroxide, and showed that the effect could be abolished by catalase.

Before considering more recent work on bacterial and mammalian cell systems, we should point out that characterization of the chemistry and biology of medium effects is important not only to clarify some radiobiological problems (see, e.g. Pollard et al. 1965), but is also critically important to the question of radiation

sterilization of food (Kesavan and Swaninathan 1971).

b) Medium Effects on Bacteria

Working with E. coli mutants unable to synthesize porphyrin-containing enzymes, Adler (1963, and work cited therein) found an after-effect of radiation in which the viability of irradiated cells decreased upon exposure to the irradiated suspending buffer. The effect was eliminated by resuspension in unirradiated buffer, or by adding catalase to the buffer, and was reproduced by resuspending the irradiated cells in unirradiated buffer containing hydrogen peroxide. Exposure of unirradiated cells to hydrogen peroxide had no effect, indicating that radiation sensitizes these catalase-negative cells to hydrogen peroxide.

Much work has been done on the anti-bacterial effect of irradiated carbohydrates, especially glucose and sucrose. Molin and Ehrenberg (1964) added glucose irradiated in solution or crystalline form to growth medium to give a final concentration of 0.5%. The bactericidal effect observed was more pronounced at lower pH, and was almost eliminated after 60 minutes exposure to catalase before inoculation. The oxygen tension was not controlled during irradiation, and therefore conditions in the Mrad dose range were esentially anoxic (Schubert 1967).

The chemistry and microbiological toxicity of

irradiated sucrose in solution has been extensively studied by Schubert and colleagues. Schubert et al. (1967) studied the disappearance of H_2O_2 formed in irradiated oxygenated and anoxic, buffered (pH = 7.0) and unbuffered (final pH = 3.2) solutions. The authors found significant growth inhibition of Salmonella typhimurium exposed to the unbuffered irradiated sucrose in growth medium after the H_2O_2 had disappeared. The growth inhibiting factor was present in both oxygenated solution and anoxic solution to which reagent H_2O_2 was added after irradiation. A less potent effect was in the anoxic solution, in which no $H_2O_2$is detectable. The inhibition decreased on storage at neutral pH. Catalase added to the sucrose-medium mixture prior to inoculation offered partial protection; the extent of protection was less when catalase was added after inoculation, and decreased with time (Schubert and Watson 1969). The authors proposed that the antibacterial effect of irradiated sucrose is due to hydroxyalkyl peroxides formed by reaction at low pH of H_2O_2 with carbonyls formed in the radiolysis of sugar.

$$H_2O_2 + RCHO \underset{\longleftarrow}{\overset{H^+}{\longrightarrow}} RCH(OH)OOH = A$$

$$RCH(OH)\text{-}OOH + RCHO \underset{\longleftarrow}{\overset{H^+}{\longrightarrow}} RCH(OH)\ OOCH(OH)\text{-}R = B$$

$$A,\ B \xrightarrow[pH=7]{H_2O} \text{hydrolytic decomposition}$$

Schubert et al. (1967) showed that the H_2O_2-glyoxal (a di-aldehyde formed in the irradiation of glucose) reaction

product was more toxic than either reactant.

The effects of irradiated medium (as opposed to constituents thereof) on unirradiated cells have been studied by Pollard and co-workers. The characteristic biological effect of irradiated medium (phosphates, chlorides, and glucose) on unirradiated E. coli was depression of biosynthetic processes. The effect, which originated in the water component, was reduced in anoxic medium, and increased as the inoculum decreased (Pollard et al. 1965). The depression in synthetic processes was shown to be due to two factors. First, a temporary halt in transcription was observed (an effect separable from DNA degradation caused by direct cellular irradiation). Secondly, the expression of preformed messenger RNA was seen to be suppressed (Pollard and Barone 1965). Frey and Pollard (1966) showed that the effect was completely protectable by catalase present during irradiation, and could be reproduced by adding reagent H_2O_2 to unirradiated medium. Using higher doses, Chopra (1969) elaborated the bactericidal action of irradiated medium, and showed a significant mutagenic effect. The effects of H_2O_2 and catalase were reproduced, although the peroxide was found to originate in the glucose component. In a later paper, Frey and Pollard (1968) showed that E. coli grown anaerobically are not affected by exposure to irradiated medium, and proposed that

the inhibitory mechanism of peroxide in the medium involves the electron transport system, depriving the cell of energy required for biosynthesis.

c) Medium Effects on Mammalian Cells

Pronounced cytotoxic effects of irradiated non-oxygenated solutions of glucose and fructose on Hela cells and mouse L cells in culture were reported by Berry et al. (1965). The cytotoxic action was not reduced by catalase, but was diminished by diluting the carbohydrate solution with irradiated water. Reagent glyoxal added in the appropriate concentration (0.22 mg/ml after 2.5 Mrad) was able to reproduce the effect, while other possible radiolytic products, formate and oxalate, were found to be considerably less effective (Hills and Berry 1967). Shaw and Hayes (1966) demonstrated a decrease in the number of human lymphocytes undergoing blast-like transformation in culture containing diluted, irradiated sucrose, and noted increased number of chromosome breaks and other aberrations in the existing mitoses.

The effects of whole growth medium irradiated to ca. 10^4 R doses on chick embryo cells in culture were studied by Levinson (1966). When cells suspended in PBS were seeded onto plates containing irradiated medium, the characteristic spreading of the cells after attachment to the

plastic was prevented. Subsequent growth on the dish, growth in agar (not dependent on spreading), and ability to support virus production were all inhibited to a comparable degree. Exposure to irradiated water produced the most potent effect, followed by growth medium, and medium with 5% serum. The presence of pyruvate during irradiation eliminated the effect, and catalase reduced it drastically. The inhibition of spreading increased as cell density decreased, and was eliminated in the presence of conditioned medium. The effect was duplicated by H_2O_2 added according to the concentration measured in irradiated medium. The author concluded that H_2O_2 or organic peroxides were responsible for the effects observed, and that pyruvate and catalase protected by removing the peroxide from the medium, while medium, serum, and conditioned medium protected through some physiological stabilization of the cell. The inhibition of spreading suggested some effect on the cell surface as the mode of action of irradiated medium.

Cells in suspension culture generally are more sensitive to their medium than cells growing attached to a substrate, since the latter can more easily condition their own microenvironment (Waymouth 1965). Therefore, the effects of stable radiation-induced products in the medium on suspension cultures are not necessarily similar to those

observed on monolayer cultures described above. Scott et al. (1966) reported a significantly longer delay in initiation of growth of L5178Y lymphoma cells inoculated in Fischer's medium exposed to less than 1 krad. Szumiel et al. (1971) using the same system elaborated on the growth inhibiting properties of irradiated medium. The authors reported phenomena indicating the radiomimetic character of irradiated medium, viz. mitotic delay, growth inhibition, and decrease in viability. The effect decreased as the post-irradiation storage time of the medium increased, and had largely disappeared in a day. The presence of cells in the medium during irradiation offered almost total protection, indicating that cells scavenge most of the harmful components. In neither of the studies on suspension cultures was there any speculation on the nature of the harmful component in irradiated medium. Recently, Rosenberg and Matthews (1973) demonstrated that exposure of L5178Y cells to irradiated medium (80 krad) prevented the volume regulating action of the plasma membrane when the cell was incubated in hypotonic medium. The effect was duplicated in H_2O_2, and reduced in the presence of catalase.

3.3 MECHANISMS OF PEROXIDE ACTION ON CELLS

Since most workers have circumstantially identified hydrogen peroxide or some mono- or di- substituted organic

peroxides as the cytotoxic principal, and many have comparable results with reagent H_2O_2, it seems reasonable to discuss the mechanism of cytotoxic action of peroxides, bearing in mind that that of irradiated medium is likely similar.

Frey and Pollard (1968) hypothesized that peroxide readily oxidizes heme iron, and in this way inactivates the cytochrome system and eliminates aerobic respiration. Indeed, the cells recovered more rapidly from the effect of irradiated medium in the presence of methylene blue, which bypasses the cytochrome system. However, this scheme is not applicable to Adler's (1963) cytochrome and catalase - negative *E. coli* H_7^- mutants which are sensitive to irradiated medium and reagent hydrogen peroxide, while added hemin, which enables the cells to metabolize aerobically, abolished the sensitivity. Peroxide attack on the cell membrane seems more plausible and generally applicable.

Peroxides generated extracellularly first interact with the cell membrane where they may readily react with anti-oxidants present, e.g. SH groups of the membrane or attached enzymes, or unsaturated lipids. Unrepaired structural damage to the plasma membrane is likely to affect vital metabolic functions through the disruption of transport mechanisms.

Levinson (1966) suggested that the inhibition of cell spreading on a plastic surface after exposure to H_2O_2 or

irradiated medium was due to cell surface damage. Wills (1970) irradiated suspensions of liver microsomes and detected lipid peroxidation occurring during the post-irradiation incubation. The main site of peroxidation was the endoplasmic reticulum. Peroxides might well be involved in this intracellular membrane destruction. The work of Rosenberg and Matthews (1973) on inhibition of volume-regulation in L5178Y cells exposed to H_2O_2 directly concerned membrane effects. The amount of H_2O_2 required to inhibit volume regulation, produced by a medium dose of about 30 krad, corresponds to the minimum dose which leads to interphase death. The process of interphase death is characterized by membrane destruction, and is thought to be caused by the oxidation of membrane SH groups (Okada 1970).

Membrane damage, however, cannot explain genetic effects induced by peroxides. Increased mutation rate has been seen in neurospora in the presence of organic peroxides (Dickey et al. 1949), and hydrogen peroxide in broth (Wagner et al. 1950). Hydrogen peroxide in broth increased the mutation rate in S. aureous (Wyss et al. 1948). Chromosomal abberations were found in human lymphocytes exposed to irradiated sucrose (Shaw and Hayes 1966), and Chopra (1969) found higher mutation and reversion rates in E. coli in irradiated minimal medium. Duplications of the last two

results were not attempted with peroxides.

Peroxides and irradiated medium have been shown to have deleterious effects on bacterial, fungal, and mammalian cells, acting as respiratory poisons, and agents causing membrane and genetic damage. It seems that peroxides act as non-specific oxidizing agents, and can induce cellular damage in many ways.

CHAPTER 4

EXPERIMENTAL

4.1 MATERIALS AND METHODS

4.1.1 Routine Procedures

a) Cell Line

Murine leukemic lymphoblasts (L5178Y), kindly provided by Dr. A.R.P. Paterson of the McEachern Laboratory, University of Alberta, were used in this study. The cells were routinely maintained in suspension culture in tightly stoppered 60 ml. bottles at 37°C, growing in Fischer's medium with 10% horse serum (Grand Island Biological Co., Berkeley, California) (Fischer and Sartorelli 1964), buffered with sodium bicarbonate and HEPES (Darzynkiewicz and Jacobson 1971). The pH was adjusted by gassing with CO_2 or exposure to air. Antibiotics were not used in the stock culture, since prolonged use encourages PPLO (pleuro-pneumonia-like organism) contamination (Eagle and Levintow 1965), and careless sterile technique (Paul 1970). However, penicillin (100 units/ml) and streptomycin (100 ug/ml) were added to short term cultures used in experiments. Medium was made up from sterile liquid concentrate, and was not sterilized further. Cell population densities were maintained between 3×10^4 cells/ml and 15×10^4 cells/ml by daily dilution in fresh medium. Stock cultures were discarded

every few months and new cultures started from a pool of cells kept frozen in liquid nitrogen (-196° C), in order to minimize alteration of cell characteristics after long-term maintenance *in vitro* (Willmer 1965, Alexander 1960).

Glassware was sterilized by dry heat (160° C for 1.5 hours) and plastic and rubber products as well as non-volatile solutions were steam sterilized. Stock cultures were checked for bacterial contamination by inspection, and by periodic plating in nutrient agar. Because antibiotics were not used, the period between infection and detection was short. Contaminated cultures were discarded.

b) Cell Growth Measurements

Cell number and relative cell size were determined in a Coulter Counter, model Fn (Coulter Electronics, Hialeh, Fla.). Cell counts were also occasionally performed using a haemocytometer (American Optical Instruments Ltd.).

c) Irradiation and Dosimetry

Plastic T-30 flasks or T-60 flasks (Falcon Plastics) containing 20 ml or 50 ml respectively of cell suspension or medium were irradiated at room temperature on an A.E.C.L. Theratron-80 Cobalt-60 Teletherapy unit (average γ energy 1.25 MeV). The flasks were irradiated from below, resting on a 0.5 cm sheet of plexiglas to ensure full build-up. The source to flask distance was sufficiently great (about 50 cm)

so that inverse square correction of the beam intensity through the flask was not important. The dose rate was measured by ferrous sulfate dosimetry used under the same irradiation conditions, and was confirmed with a Victoreen sub-standard R-meter.

d) Assays for Cell Survival

Two methods were used to determine the fraction of a cell population retaining its viability after treatment. The first, and most often used, was a modification of Chu and Fischer's (1968) soft agar colony method (Kim 1971). A 0.8 ml aliquot of some known dilution of cell suspension was added to 7.2 ml of twice concentrated growth medium supplemented by 10% (volume/final volume) conditioned medium (CM). An equal volume of liquid 0.28% Noble agar was added. The suspension was mixed by pipetting several times, dispensed into four 12 x 75 mm culture tubes, and stoppered. The tubes were set in cold water for several minutes to set the agar suspension, then placed at 37° C for 8 - 12 days, at which time the visible colonies (those containing more than 50 cells) were scored by microscope (figure 7).

Sufficient cells were "plated" to produce 10 to 100 colonies per tube. The plating efficiency (P.E. = percentage of cells which grow into colonies) for control cultures ranged from 50% to 80%. The presence of large numbers of non-viable

Figure 7. L5178Y cell colonies in soft agar.

cells did not affect plating efficiency. Conditioned medium, the supernatant medium taken from 10 day - old cultures, was included to condition the medium and facilitate growth at low cell densities.

The second method, that of growth curve extrapolation (Alexander and Mikulski 1960, Dewey et al. 1963), involved determining from the post-treatment growth curve the fraction of treated cells with respect to control cells which reattain control growth. The growth inhibition measured in this way represents delays in cell progression as well as cell death.

4.1.2 Medium Irradiation Experiments

a) Design of the Medium Irradiation Experiment

The protocol for the experiment determining the effect on cells incubated in irradiated medium is illustrated in figure 8. Irradiation was carried out as described above. Some time after irradiation (inoculation time), cells from early log-phase were resuspended in the irradiated medium (inoculation), set to incubate at the incubation temperature, and finally resuspended in fresh medium. Plating and growth measurements were carried out at this point.

b) Medium

The media used in the irradiation studies were Fischer's medium and saline. The former was used with or

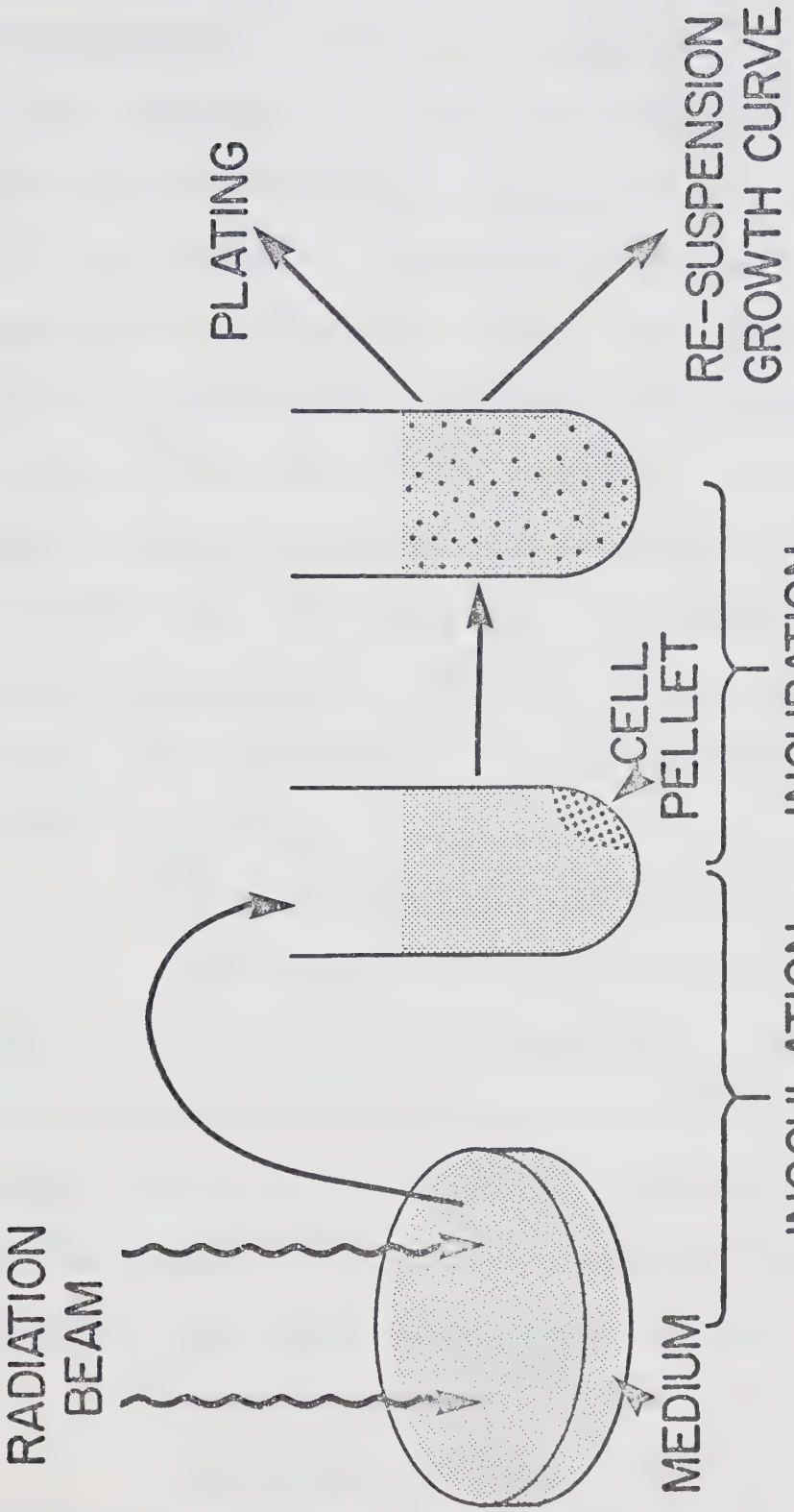


Figure 8. Protocol for medium irradiation experiments

without the normal 10% complement of horse serum (referred to respectively as whole or defined medium), and antibiotics. An extra supplement of 10% conditioned medium was included for nutritional reasons in some early experiments involving long incubations in irradiated medium (see section d). Medium was irradiated at slightly lower pH than physiological in order to accommodate the rise in pH in newly resuspended cultures (there was no pH change due to irradiation). The saline used for irradiation was Dulbecco's phosphate-buffered saline (PBS, pH 7.4). This medium does not support cellular metabolism at 37°C, therefore saline incubation was at 0°C. The compositions of the irradiated media are found in appendix I.

c) Resuspension Procedure

Cell suspensions were resuspended in irradiated medium (inoculation) and subsequently in fresh medium by spinning at 1000 rpm for 5 minutes in 16 x 100 mm stoppered plastic culture tubes, discarding the supernatant, and adding the new medium. The cell suspension was dispensed into duplicate tubes after resuspension in fresh medium.

d) Length of Incubation

After preliminary experiments involving essentially indefinite incubation in irradiated medium, the possibility of growth-inhibition due to radiation-induced medium depletion

was removed by limiting the time of incubation. It was found that 1 hour incubation in irradiated medium followed by resuspension in fresh medium produced 85% of the damage due to indefinite incubation, and this condition was therefore adapted as standard.

4.1.3 Incubation in Hydrogen Peroxide-containing Medium

a) Hydrogen Peroxide Incubation

The procedure for H_2O_2 incubation experiments was the same as that for incubation in irradiated medium. Hydrogen peroxide (30%, Fisher "Certified") was diluted in cold PBS, and added at the desired final concentration to the medium of incubation minutes before inoculation.

b) Hydrogen Peroxide Determination

Hydrogen peroxide was measured by the starch-iodide method of Savage (1951). In this assay the iodine formed by H_2O_2 oxidation of iodide complexes with starch to form a blue-absorbing compound which can be measured colorimetrically. To 5.0 ml H_2O_2 solution was added 0.5 ml each of 1M fresh potassium iodide, 1 mM ammonium molybdate in 1N sulfuric acid, and 5% fresh starch solution. The product was monitored at 600 nm, the absorption peak of the starch-iodine complex. A calibration curve was made using known concentrations of H_2O_2 in water. It was possible to

measure reproducibly H_2O_2 levels of 0.05 µg/ml to >1.2 µg/ml by this method.

c) Catalase Activity Assay

Beef liver catalase, obtained from Sigma Chemical Co., St. Louis, Mo., was used for enzymatic decomposition of hydrogen peroxide. The assay used for determining catalase activity was that outlined in the Sigma catalogue. The crystalline suspension of catalase was diluted 5000 times in distilled water, and 0.1 ml added to 2.9 ml of H_2O_2 at ca. 15 µmole/ml (600 µg/ml), in a silica spectrophotometer cuvette. The loss of H_2O_2 was monitored as a decrease in O.D. at 240 nm.

The enzyme activity in the reaction mixture is defined as follows: 1 unit causes the decomposition of 1 µmole/minute H_2O_2 at an H_2O_2 concentration of 10 µmole/ml. This corresponds to a decrease in OD_{240} from .450 to .435. The catalase activity was determined to be 300,000 units/ml. The activity of a dilute solution (1200 units/ml) fell to half its original value after one day, therefore dilute solutions were always used within an hour of preparation.

4.2 RESULTS

4.2.1 Cellular Radiation Response

The radiation response of L5178Y cells was examined

to characterize their radiosensitivity, and, incidentally, to monitor that radiosensitivity over the course of these studies. Cell survival in soft agar, and post-irradiation growth characteristics were studied in the dose range which gives rise to reproductive cell death. The radiosensitivity of the ribosome-polysome system was briefly investigated as an indication of damage to sub-cellular components in cells subjected to lethal and supralethal radiation doses.

a) Agar Colony Survival

Cells from log phase were irradiated in growth medium at various times over a 15 month period. Their radiosensitivity, determined by cell survival in soft agar, was unchanged during this time. Figure 9, using data from several experiments, shows a Do of 100 rads, and an extrapolation number of 2.0. Survival was not dependent on cell concentration.

b) Post-irradiation Growth

Figure 10 illustrates typical cell growth characteristics following irradiation. The mitotic delay for cells irradiated to 400 rads is shown, and can be seen to be about 6 hours. This delay was found to increase with dose. For reasons of clarity, other data points have been omitted. Almost all cells in the irradiated population underwent at least one normal cycle at the control rate. The relative cell number dropped with respect to the control, i.e. cell

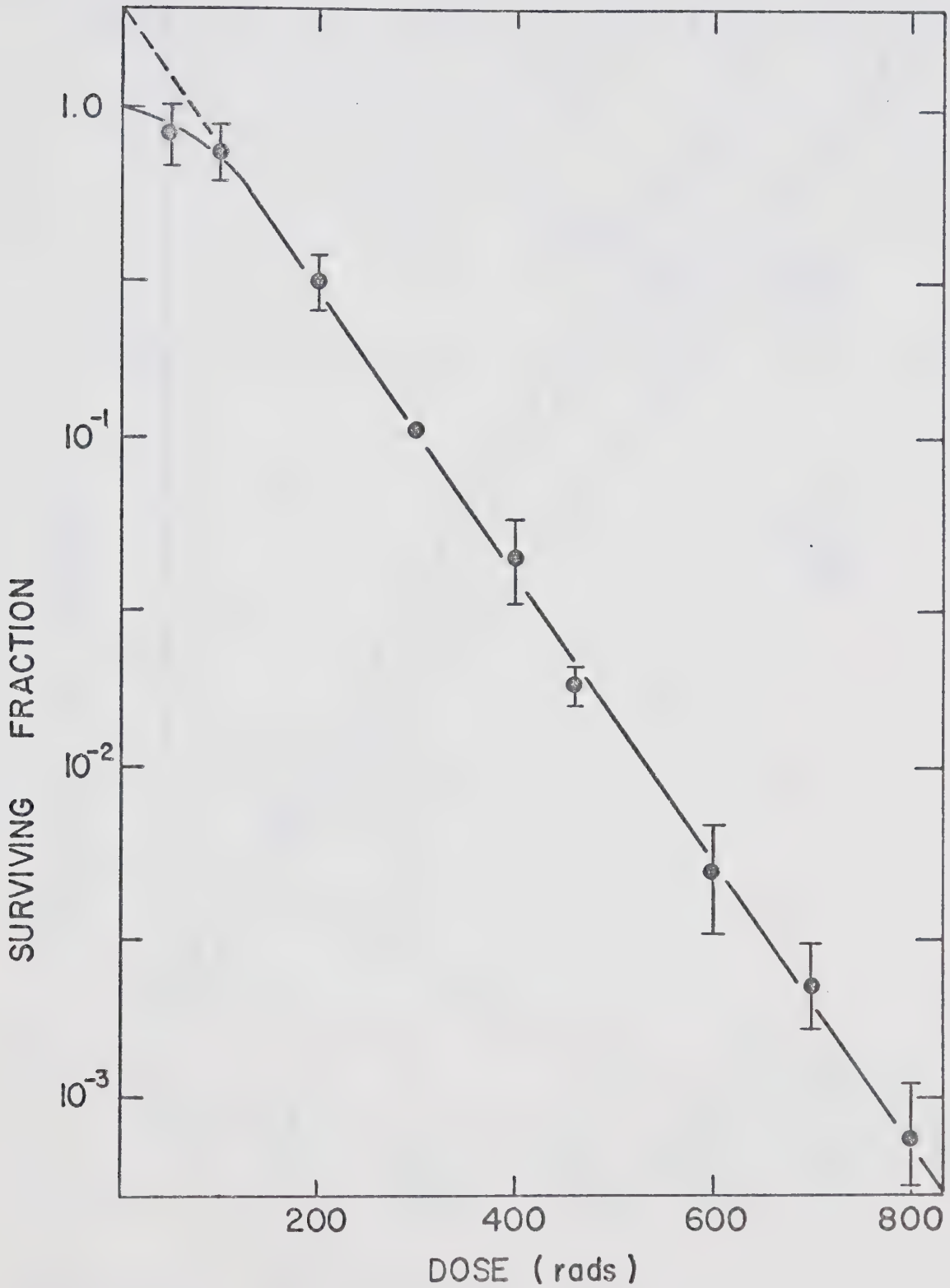


Figure 9. Survival curve of L5178Y cells. Reproductive integrity determined by colony formation in agar. Standard errors of pooled data indicated. D_o = 100 rads, n = 2.0.

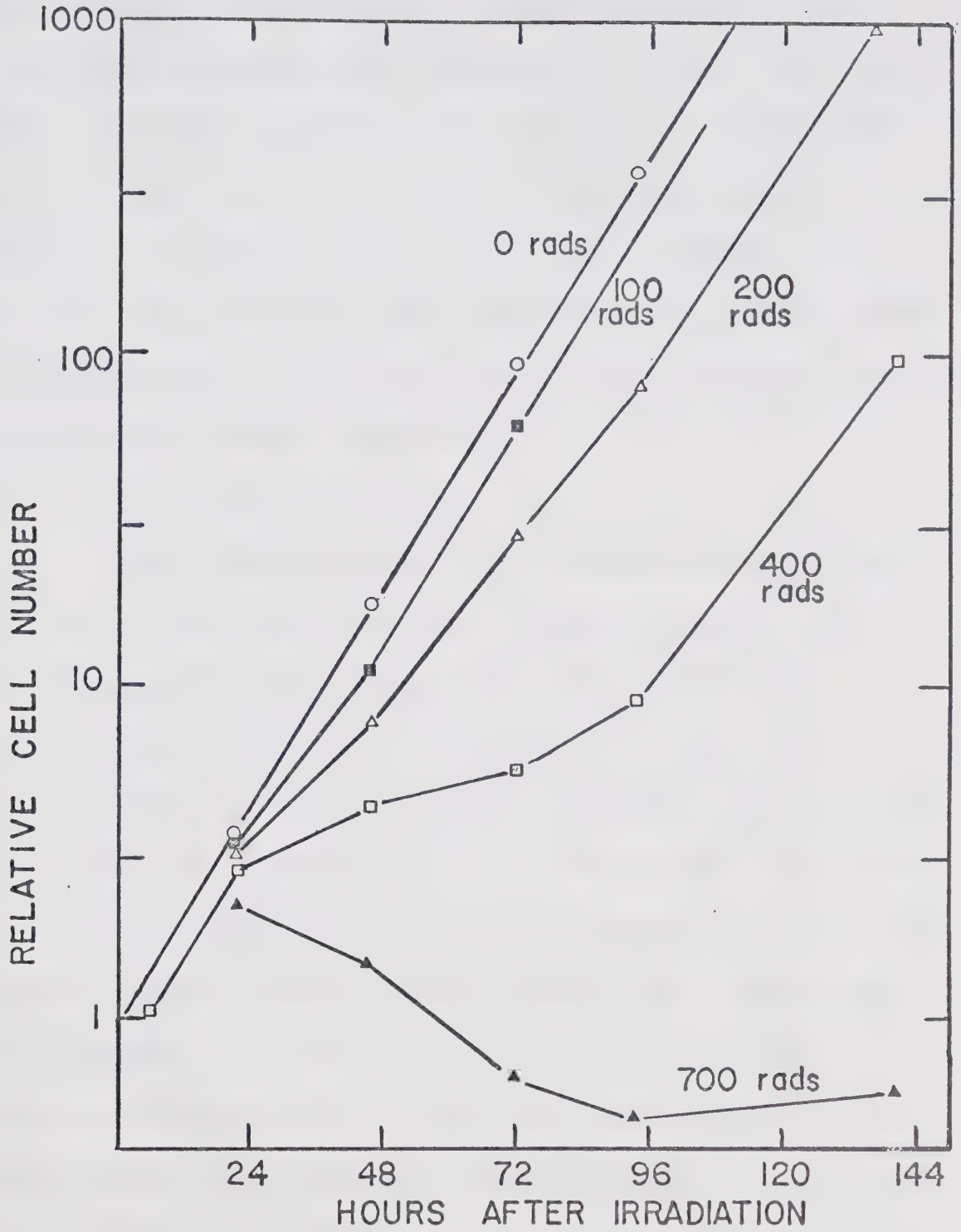


Figure 10. Post-irradiation growth.

death occurred, only after one or more divisions. Growth in the surviving population attained the control rate after several days, and comparison with the control growth curve shows that this fraction is 2.0% for 400 rads (after correction for mitotic lag). This figure, slightly lower than the colony survival value represents cell death as well as retarded growth of surviving cells, which does not show in colony survival curves (figure 9).

c) Cell Size Distribution

Giant cells appeared in irradiated culture after one cycle. These cells are more correctly called "large cells" because they are those which counted above an arbitrary size threshold in the Coulter Counter. The population of large cells includes true giant cells, that is, those cells which metabolize but do not divide. The relative number of large cells in the population exposed to 200 rads reached a maximum of 25% several cycles after irradiation, then declined to the control level as the culture re-attained control growth. A giant cell which appears after several normal post-irradiation cycles causes an end to its pedigree. This phenomenon was seen in agar colony growth as an abortive colony. These contained less than 50 cells, and were not scored.

Post-irradiation growth curves were identical for

cells growing in the medium in which they were irradiated and those which were resuspended in fresh medium after irradiation. This indicates that irradiation at doses less than 1000 rads does not impair the nutritional "carrying capacity" of the suspending medium, and also that there is no observable harmful effect to the cells originating in the medium at these doses.

d) Sedimentation Analysis of Ribosones and Polysomes

Introduction :

The effects of cellular radiation on sedimentation profiles of polysomes were investigated as an indication of sub-cellular damage. Radiation effects on the protein biosynthetic system, (for example depressed DNA synthesis, RNA synthesis, and depressed or stimulated protein synthesis), may be expected to be expressed as changes in the structure or activity of cellular polysomes.

Degradation of ribosomes to ribosomal sub-units have been observed by sedimentation analysis in irradiated bacteria (Kucan 1966, Tamaoki et al. 1968). Whole-body irradiation in rats caused a shift in the polysome sedimentation profile to the monomer (monoribosome) region in regenerating liver (Yatvin and Lathrop 1966) and thymus (Saroja et al. 1971). Irradiation of root meristem cells caused a shift of protein synthetic activity to smaller polyribosomal aggregates; however no shift in the polysome

population distribution was observed in the sedimentation profile (Kovacs and Van't Hof 1972).

Materials and methods:

Cells were irradiated to 500 rads (lethal dose) and 3000 rads (supralethal dose), and incubated for 1 to 2 hours at 37°C. They were then resuspended in buffer containing 20 mM tris-HCl (pH 7.4), 5 mM Mg^{2+}, 100 mM K^{+}, 6 mM 2 - mercaptoethanol, 0.5 mM dithioerythritol, and 0.1% Triton X-100 (Gielkens et al. 1971). Lysis was complete after 20 minute incubation at 0° C. Nuclei and cell debris were removed by spinning at 2000g for 10 minutes, and 0.6 to 0.7 ml of the supernatant layered carefully on linear 10 - 30% sucrose gradients. The centrifugation was performed at 4° C using the SB-283 rotor in the IEC B-60 ultra-centrifuge for 60 minutes (for polysome analysis) or 120 minutes (for sub-unit analysis), at 39,000 rpm. The gradients were removed from the top of the tube by displacement with 50% sucrose pumped into the bottom, and the effluent fed into a flow cell for continuous recording of absorbance at 260 nm in the Beckman DB-G spectrophotometer connected to an X-t strip chart recorder.

Results:

No detectable radiation induced effect was seen on polysome/ribosome or ribosome/sub-unit population

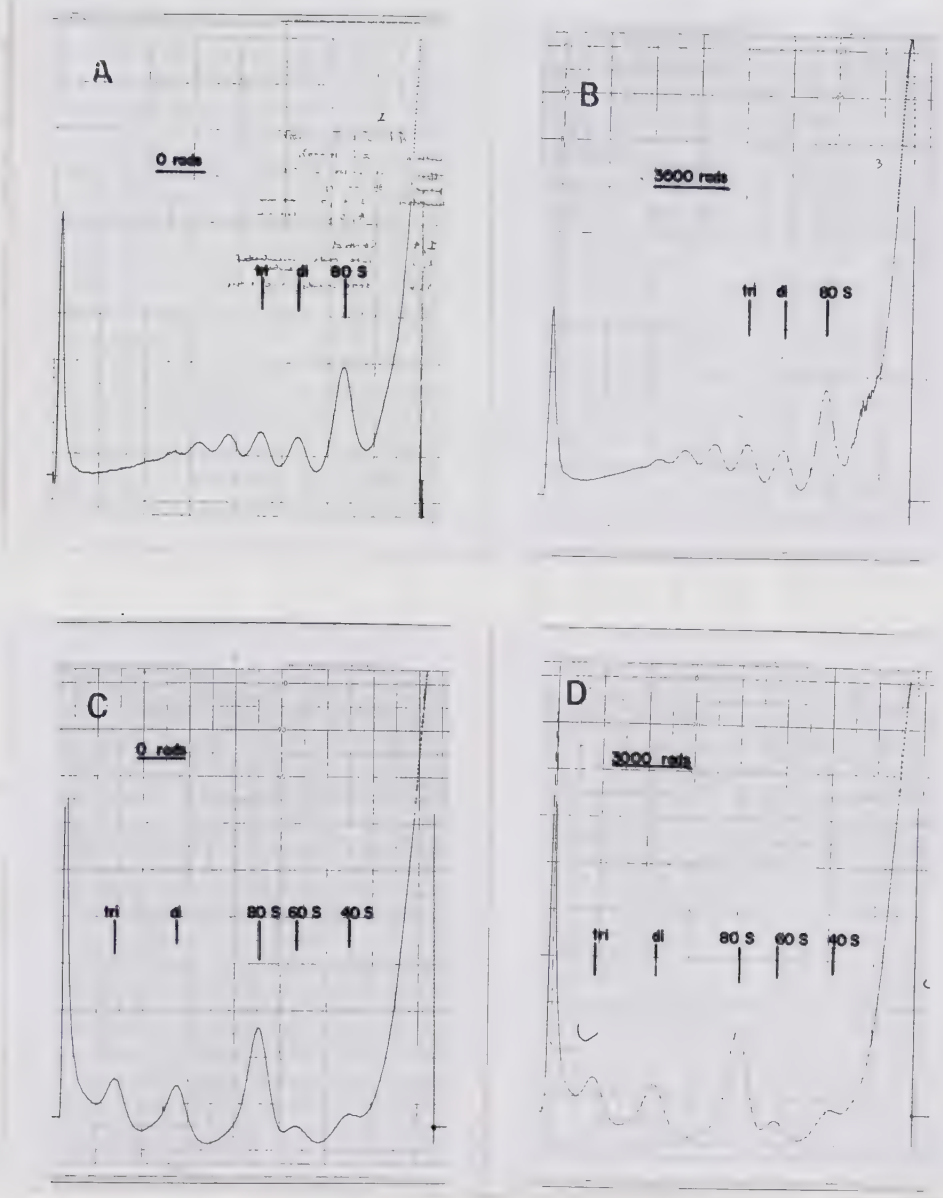


Figure 11. Ribosome sucrose density gradient sedimentation profiles. A and B: polysome profiles. 80S, monomer; di, dimer; tri, trimer. C and D: sub-unit profiles. 40S, small sub-unit; 60S, large sub-unit. Ordinate: full-scale = 1.0 $O.D._{260}$. Direction of sedimentation: right to left.

distributions in sedimentation profiles, shown in figure 11. It should be emphasized that this assay can indicate only gross changes in ribosome structure or drastic shifts in ribosome/polysome populations due to whole-cell irradiation. It is not a sensitive test of ribosomal or translational radiosensitivity.

4.2.2 Medium Irradiation

In these experiments it was intended to describe and analyse any observed effects of the incubation of cells in irradiated medium.

For the purposes of the descriptive experiments, it was sufficient to attribute the damage to the cells to a long-lived radio-chemical product (or products) toxic to cells, and a radiation-induced nutritional impairment of the medium. The former effect would be expected to decrease with inoculation time, as the radio-product decayed or underwent reactions, and would be expected to cause both growth inhibition and cell death. Nutritional impairment must occur in the serum (or possibly, conditioned medium) component of the medium; small molecules are not appreciably altered at the doses delivered, but proteins at low concentration may be damaged. Even so, the loss of active serum (or CM) protein molecules will impair cell growth

only if the active protein, rather than its degradation products, is required in cellular metabolism. Since cells grow (more slowly) in protein-free medium, a radiation-induced nutritional impairment can only retard growth. This effect should remain constant with inoculation time, since there can be no repair occurring in the medium, and will only be seen for long incubations.

With these simplified mechanisms in mind, the effect of irradiated medium on cells was described in terms of dose response, kinetics, dependence on culture conditions, and dependence on the composition of the medium.

The analysis of the effect was undertaken by adding antagonists to diminish or abolish it, and then by attempting to mimic the effect without radiation.

a) Cellular Growth after Incubation in Irradiated Medium

Dose response:

The effect on cell growth and plating efficiency of incubation in medium irradiated to doses in the kilorad range is shown in figure 12. The medium was inoculated with 5×10^4 cells/ml 1 minute after irradiation, and incubated for 1 hour at 37°C. Several features can be seen in cell growth after incubation which distinguishes it from growth after cellular irradiation. Growth inhibition occurs promptly, and there is extensive cellular disintegration visible 4 to 6

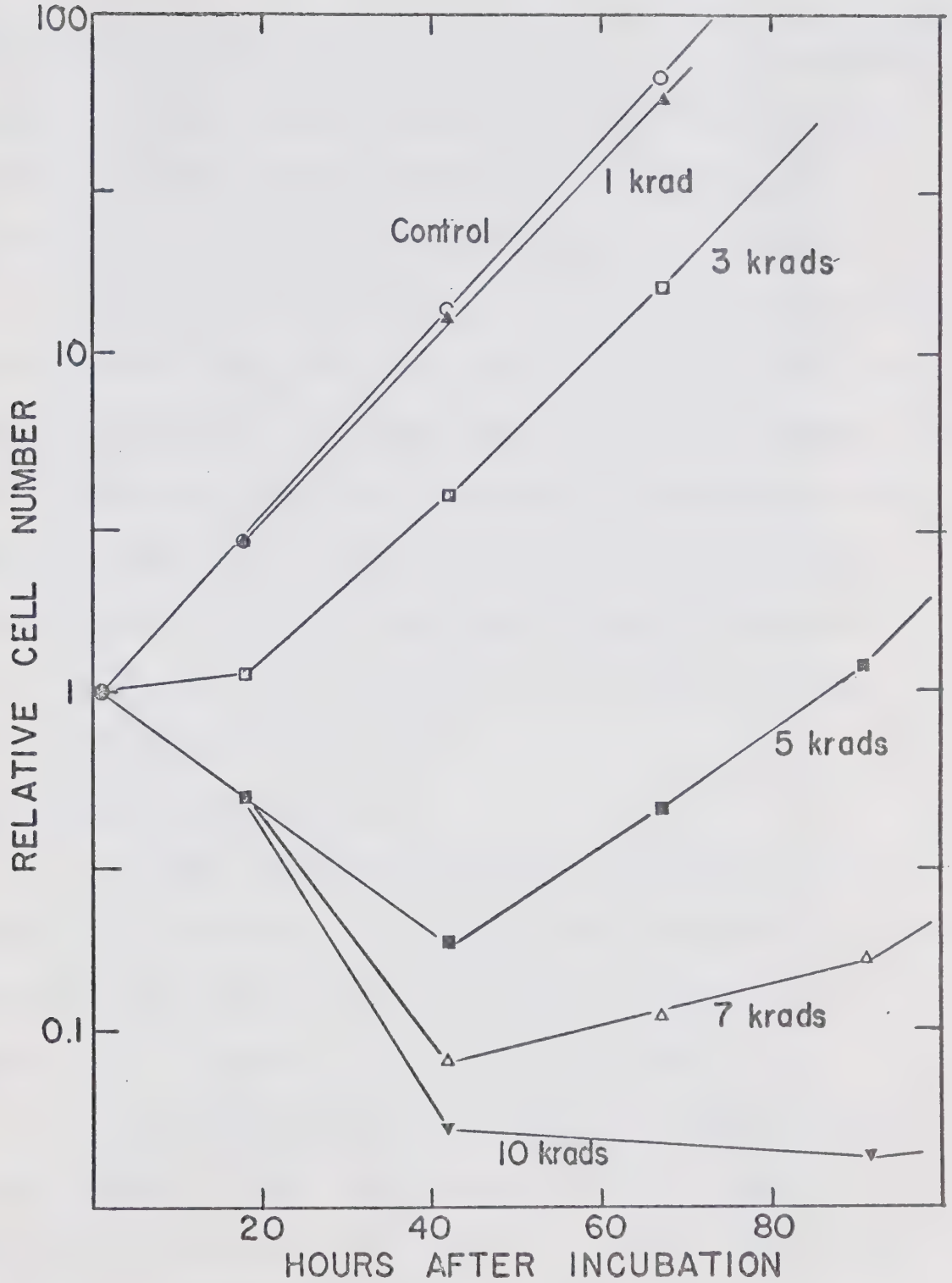


Figure 12. Post-incubation growth: function of dose to medium

hours after incubation at low-survival (< 10%) dose points (figure 13). Cells regain growth at the control rate more quickly. Survival determined by agar colony formation is comparable to that obtained from the growth curves.

The dose response is similar to that of irradiated cells. Little damage was expressed at low doses, and survival decreased progressively as the medium dose was increased. The dose to the inoculated medium required to cause comparable death in irradiated cultures is about 10 times greater. Damage incurred to cells incubated in medium irradiated to 1 krad is insignificant compared to that resulting from cellular irradiation at 1 krad.

Inoculation time dependence:

As the time interval between irradiation and inoculation increased, growth impairment decreased. Figure 14 shows this trend for 7×10^4 cells/ml incubated for 2 hours at 37° C in medium irradiated to 5 krad.

This effect can be described as a superposition of two factors. First, as discussed earlier, there is a decay of the toxic radiation product. This may be caused either by spontaneous degradation, or by chemical or enzymatic reaction with components in the medium. The former mechanism was discounted by results indicating no inoculation time dependence for cell viability after incubation in PBS.

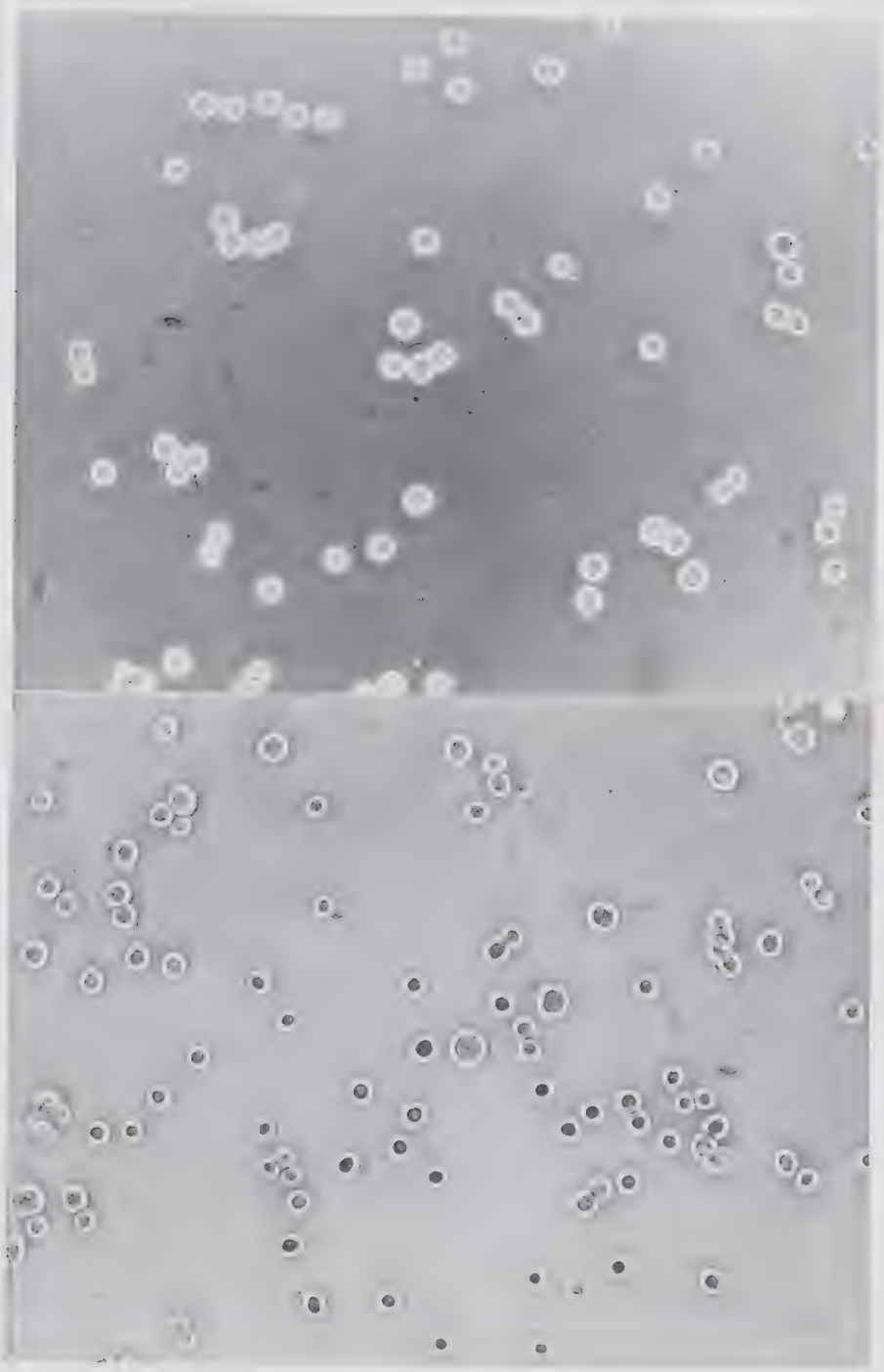


Figure 13. L5178Y cells after incubation in irradiated medium. Disintegrating cells and giant cells seen in the population exposed to irradiated medium (bottom) are not present in the control culture (top). Phase contrast X240.

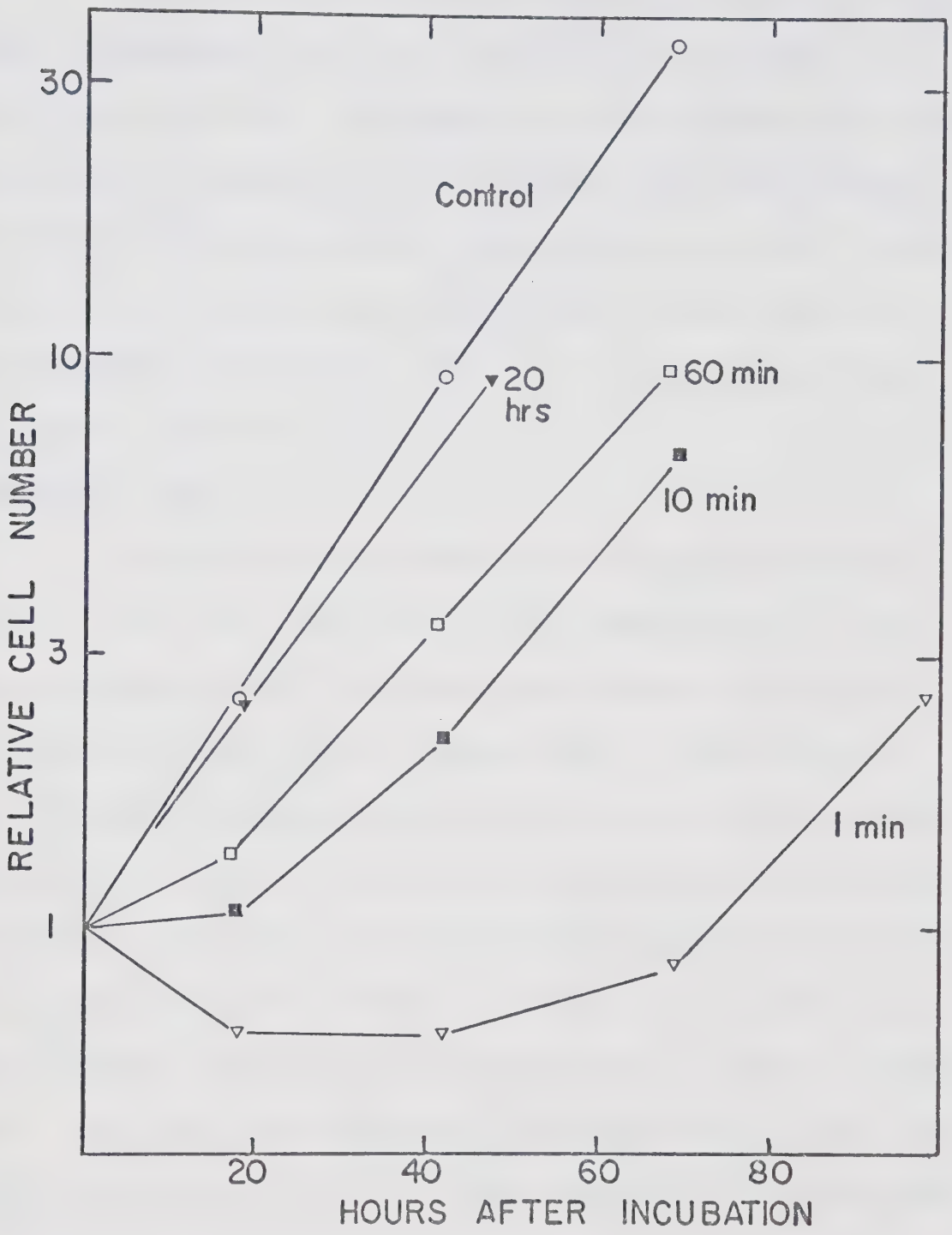


Figure 14. Post-incubation growth: function of inoculation time.

The second factor is the cellular response to the toxic radio-product, i.e. cell survival as a function of product concentration. It might be naively assumed that both product concentration vs. time in medium, and cell survival vs. product concentration are exponential functions. In this case, the curve of log cell survival vs. inoculation time will be convex increasing. This is in crude agreement with observation (see figure 16).

Incubation time:

In a preliminary experiment the time-course of action of the toxic radio-product was investigated. Cells were incubated at 37° C in irradiated medium (10 krad) for 5 minutes and longer before resuspension in fresh medium. Cell viability was determined by growth curve extrapolation (figure 15a). Figure 15 (b) shows that most of the cellular inactivation resulting from essentially indefinite incubation had occurred after 20 minutes incubation. On the basis of these results, 60 minutes was chosen as the standard incubation time. This condition eliminates any effect due to impairment of nutritive capacity of the irradiated medium, as discussed earlier, and ensures identical initial growth environments for control and treated cells.

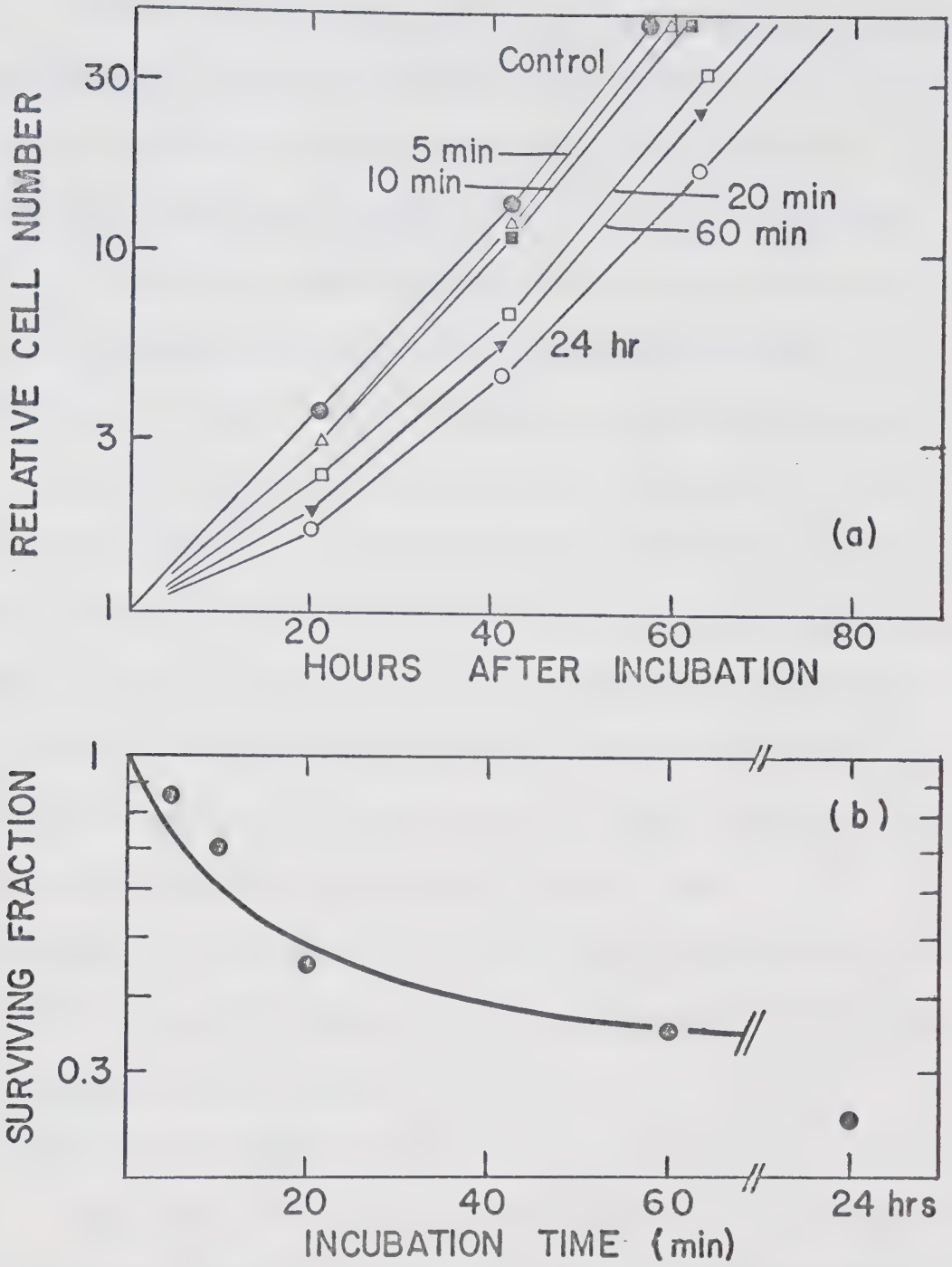


Figure 15. Incubation time characteristics.
a) Post-incubation growth as a function incubation time. b) Surviving fraction determined by growth curve extrapolation, vs. incubation time. Medium dose = 10 krads.

Temperature of incubation:

Incubation of cells in irradiated medium at reduced temperatures will have less effect than incubation at 37°C if the damage caused by the radio-product is dependent on cellular metabolism (as opposed, for instance, to damage caused by irreversible modification of essential cellular material by chemical reaction with the radio-product). To test this possibility, 4×10^4 cells/ml were incubated in medium irradiated to 5 krad at 0°C, 24°C, and 37°C. It is expected that cellular metabolism is slowed at 24°C, and virtually stopped at 0°C. Survival after incubation at these temperatures was ca. 10% for 0°C incubation, compared to about 1% for the higher temperatures. (The first figure is probably low because the culture was at room temperature for 5 to 10 minutes during inoculation, mixing, and equilibration in the ice bath.) These data suggest that the mechanism of cellular damage by the radioproduct is at least partly metabolism dependent.

Constituents of irradiated medium:

Cells were incubated in PBS, serum-less (protein-free) "defined" medium, and "whole" medium (containing 10% horse serum), to investigate the origin of the toxic radiation product.

Scott et al. (1966) pointed out that cells incubated in irradiated medium were subject to different

degrees of growth inhibition, depending on the batch of serum. This fact implicates protein in the medium, either as the component in which the radiation product originates, or that which influences its degradation. The results of a number of experiments, presented in table V, clearly indicate that the toxic radiation product originated in the saline component of medium. Furthermore, the serum acted as a protector; different batches of serum protected to different extents. Considerable variability in response to incubation in irradiated medium containing different batches of serum was seen. Figure 16 shows that the increase in cell survival with inoculation time is quicker in the medium (batch "A") providing more efficient protection than another batch ("B"). As mentioned earlier, incubation in irradiated PBS ("C") did not show the characteristic inoculation time dependence. This dependence in protein-containing medium is consistent with a protective mechanism of chemical or enzymatic degradation of the toxic product. The rate of degradation (and consequently the extent of "protection" with respect to incubation in defined medium)depends on the amount or activity of the protective agent in the serum.

b) Effect of Catalase Addition to Irradiated Medium

The observation of a more potent cytotoxic effect due to incubation in irradiated PBS than in medium points to

TABLE V

Cell viability following 1 hour incubation in irradiated medium as a function of serum concentration, and in irradiated PBS.

Dose (krad)	Incubation Temperature (°C)	% Survival			
		Fischer's Medium with % Serum			PBS
		30	10	0	
10	0°C		7.5		1.8
10	0°C		8.1		0.6
5	0°C	76	15	1.5	
10	0°C		23	4.5	2.9

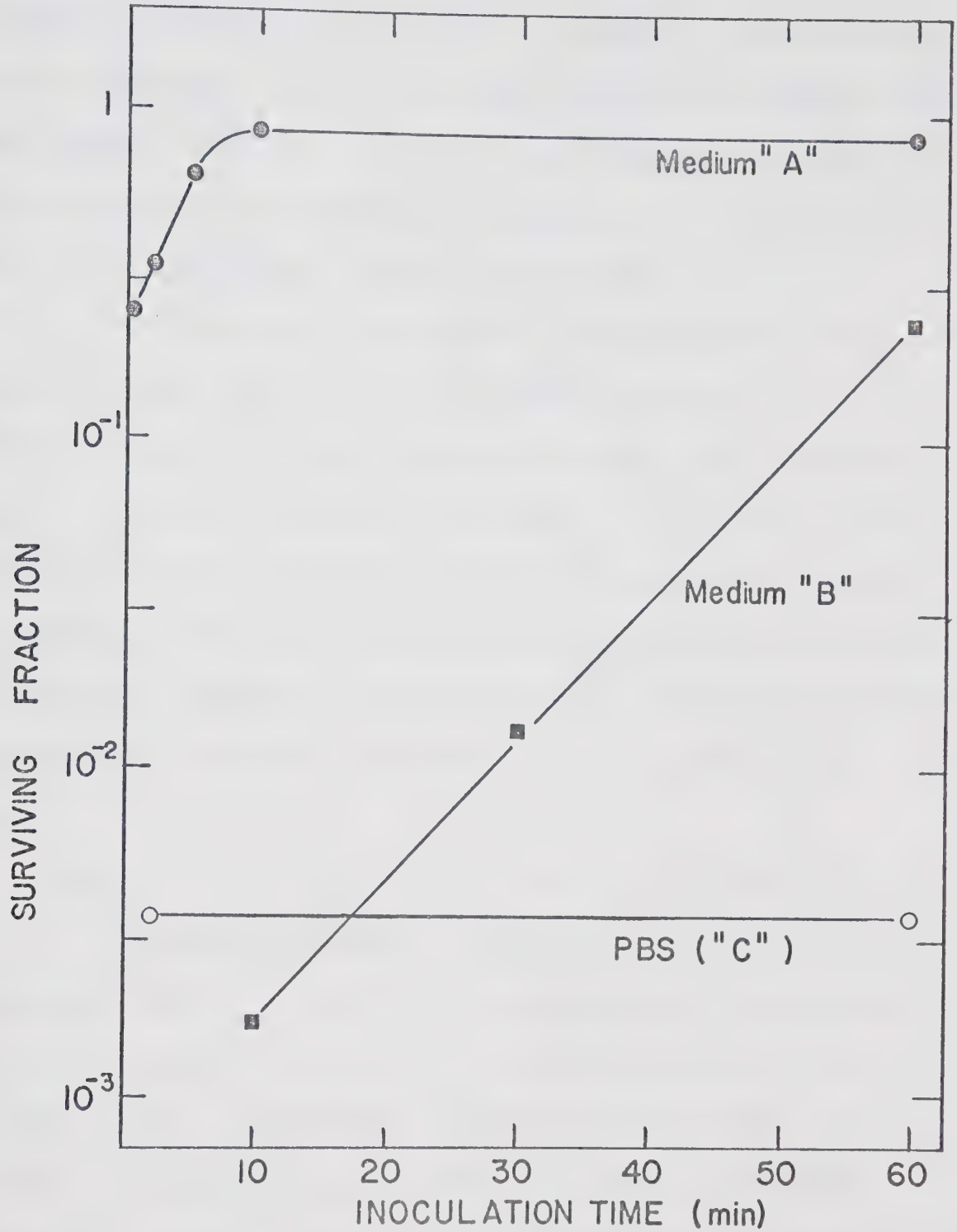


Figure 16. Variability in viability and inoculation time dependence according to the serum batch used in whole media, "A" and "B". PBS ("C") shows no inoculation time dependence.

a radiolytic product of water (or, certainly, aqueous saline) as the radio-toxic agent. Of these, hydrogen peroxide is one likely agent. In order to test this, catalase was added to the irradiated medium during the incubation of cells and the extent of the cytotoxic effect was examined.

Catalase is a heme enzyme, molecular weight ca. 250,000 daltons, found in all organisms employing the cytochrome system in their respiratory chain, viz. animals, aerobic bacteria, and plants (Saunders et al. 1964). Its principal biological function is catalysing the decomposition of peroxides (mainly and most effectively H_2O_2) which accumulate in oxidative reactions. Crystalline water suspensions of the enzyme rapidly decompose hydrogen peroxide to water and oxygen

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \text{ , } k=3 \times 10^7 M^{-1} sec^{-1}$$

Catalase was added to suspensions of cells in irradiated medium (5 krad) 1 to 2 minutes after inoculation, at a final concentration of 0, 1, 3, and 10 units/ml. Colony survival results in table VI A indicate significant, but incomplete protection by catalase. In order to determine whether the protection was, in fact incomplete, or simply too late, catalase (10 units/ml) was added to irradiated whole medium, defined medium, and PBS at various times relative to inoculation. Results presented in Table VI B

TABLE VI

% Viability after incubation in irradiated medium: protection by catalase

A

Medium	Dose (krad)	Incubation Conditions	Catalase Concentration (units/ml) Added 1-2 minutes after inoculation			
			0	1	3	10
Fischer's Medium + 10% Serum	5	60 min at 37°C	5	60	80	70

B

Medium	Dose (krad)	Incubation Conditions	Time of catalase (10 units/ml) addition (before) or after inoculation (min)			
			(1.0)	0.5	2.0	no catalase
Fischer's Medium + 10% serum	10	60 min at 0°C	105			23
Fischer's Medium (no serum)	10	60 min at 0°C	96			4.5
PBS	10	60 min at 0°C	12	7	3.8	2.9

show an interesting difference between incubation in medium (with or without serum) and in PBS vis-a-vis the protection offered by catalase. In particular, 10 units/ml catalase (which will digest 63% of the available H_2O_2 per minute) added 1 minute before inoculation (1.5 - 2 minutes after irradiation) afforded complete protection to cells incubated in whole and defined medium, while the protection of cells incubated in PBS is significant but limited. Since the damage due to incubation in medium is protectable by catalase, and at a rate which suggests catalatic action (H_2O_2 as subtrate) rather than the slower peroxidatic action (ROOH as substrate) (Chance 1949), hydrogen peroxide must be considered as the damaging component. However, the data for catalase protection in irradiated PBS suggest the presence of some other component(s) which either possesses cytotoxic capabilities, or is able to inactivate the catalase. These possibilities are investigated in the following section.

4.2.3 Incubation in Medium Containing H_2O_2

a) Dose and Temperature Characteristics

The yield of hydrogen peroxide in pure, aerated water is G = 0.75, or 0.25 µg/ml after 10 krad. Savage (1951), and Frey and Pollard (1966) measured 0.7 - 0.8 µg/ml and 0.4 µg/ml H_2O_2 respectively in water irradiated to

10 krad. Cells were incubated in medium containing H_2O_2 in the 0.1 - 1 µg/ml range. Figure 17 shows cell survival results (growth-curve extrapolation and plating data taken from different experiments) following incubation for 60 minutes in whole medium and PBS at 0°C and 37°C. Slightly greater effect is seen after incubation in PBS compared to whole medium at 0°C. Incubation at 37°C is much more destructive than at 0°C. It should be mentioned in connection with the observed temperature dependence that the usual experimental protocol (followed in all irradiation experiments) called for inoculation at room temperature before setting at 0°C after 1 to 2 minutes. Inoculation for the dose-effect curve in PBS was done in cold medium, therefore cell survival might be overestimated.

b) Inoculation and Incubation Time Dependence

Cell survival after 60 minute incubation at 37°C in whole medium containing 0.4 µg/ml H_2O_2 increased from 0.5% to 4.1% as the inoculation time increased from 0.5 minutes to 60 minutes. This increase was not seen in PBS. As in the case of irradiated medium, the inoculation time dependence in whole medium is taken as evidence of an induced degradation of H_2O_2.

The dependence of cell survival on incubation time is shown in figure 18, for incubation in whole medium with

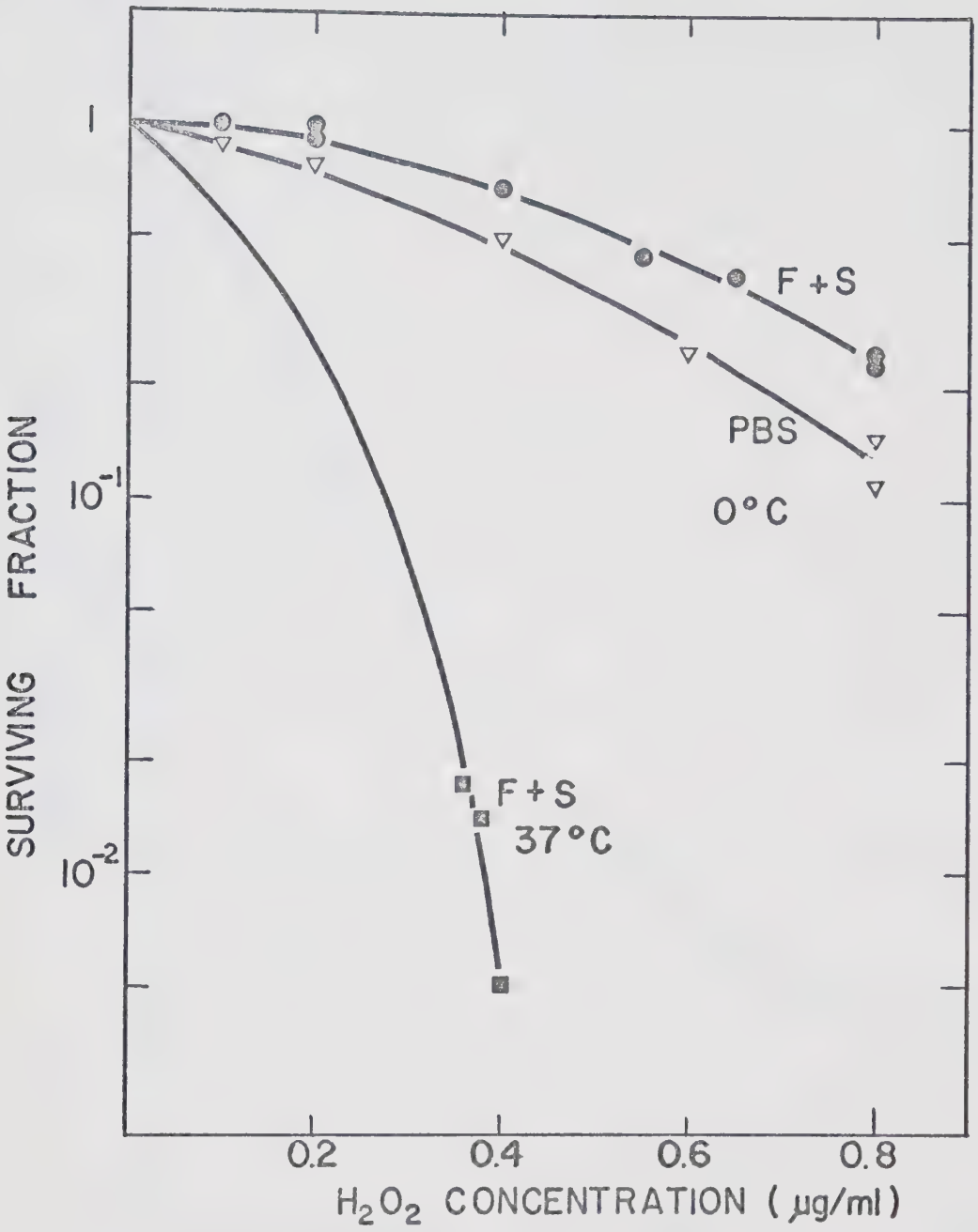


Figure 17. Cell viability after incubation in whole medium (F + S) at 37°C and 0°C, and PBS at 0°C, as a function of H_2O_2 concentration.

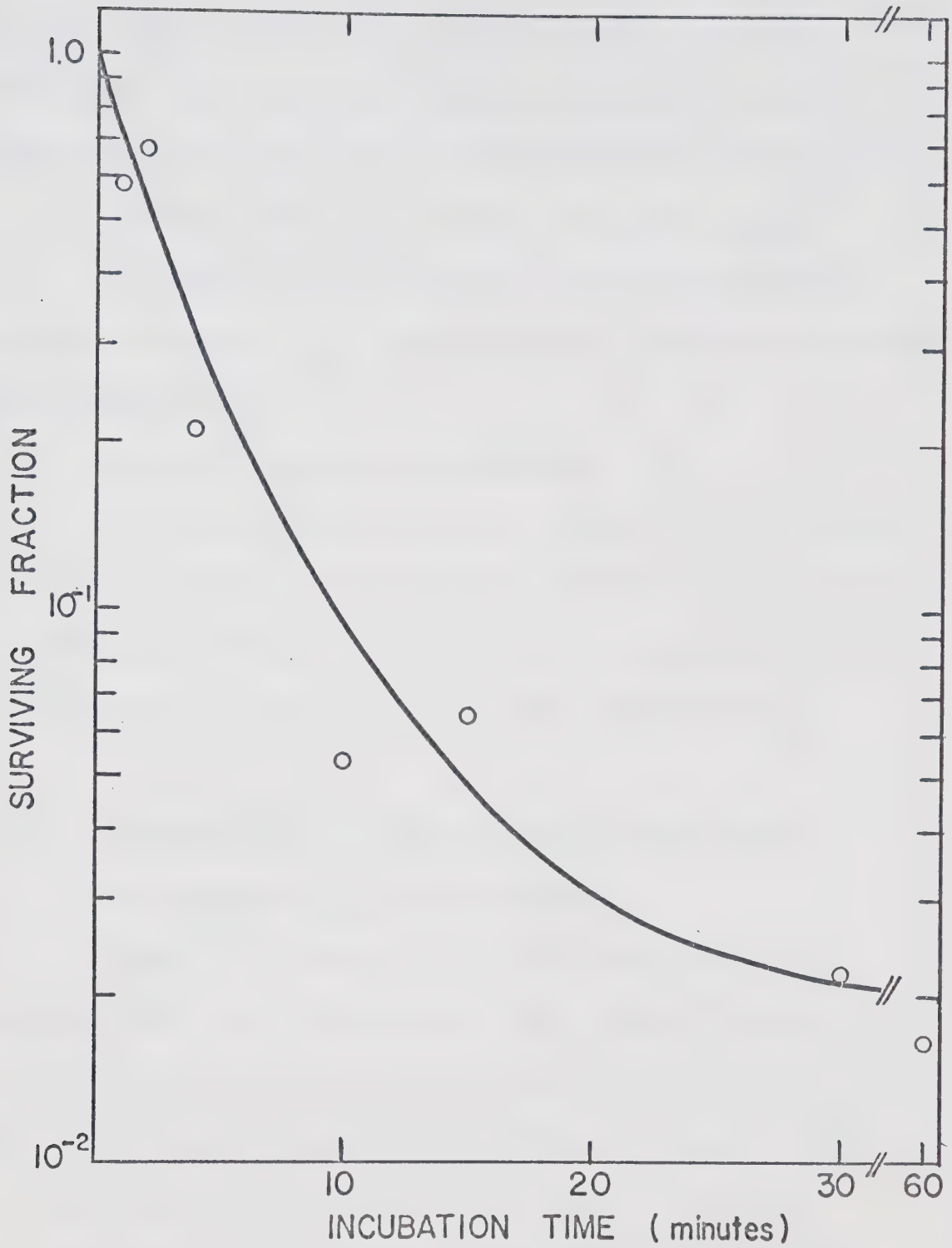


Figure 18. Cell viability as a function of incubation time in whole medium containing 0.37 μg/ml H_2O_2.

0.37 µg/ml H_2O_2 at 37°C. The action of H_2O_2 is rapid, levelling off at 30 minutes; this is comparable to the corresponding experiment with irradiated medium (figure 15b).

c) Catalase Addition to Medium Containing H_2O_2

The addition of 10 units/ml catalase to medium containing 0.8 µg/ml H_2O_2 1 minute before inoculation offered complete protection.

d) Hydrogen Peroxide Measurements

The iodide-starch method of Savage (1951) was used to measure H_2O_2 formed in irradiated PBS and water (doubly-distilled, deionized, pH ≈ 6.0). The values shown in figure 19 are reasonably consistant with those quoted above.

4.2.4 Incubation in Irradiated Medium, and Medium Containing H_2O_2 : a Comparison

Figure 20 compares the incubation growth characteristics (cell multiplicity with respect to control) for cells incubated in irradiated medium (10 krad), and medium containing 0.65 µg/ml H_2O_2 (0.8 µg/ml H_2O_2 in PBS) for 60 minutes at 0°C. Figure 20 (a) shows that these conditions cause similar growth impairment in whole and defined Fischer's medium. Also, the fact that addition of catalase to irradiated Fischer's medium provides complete protection (table VI and figure 20 a) suggests that the

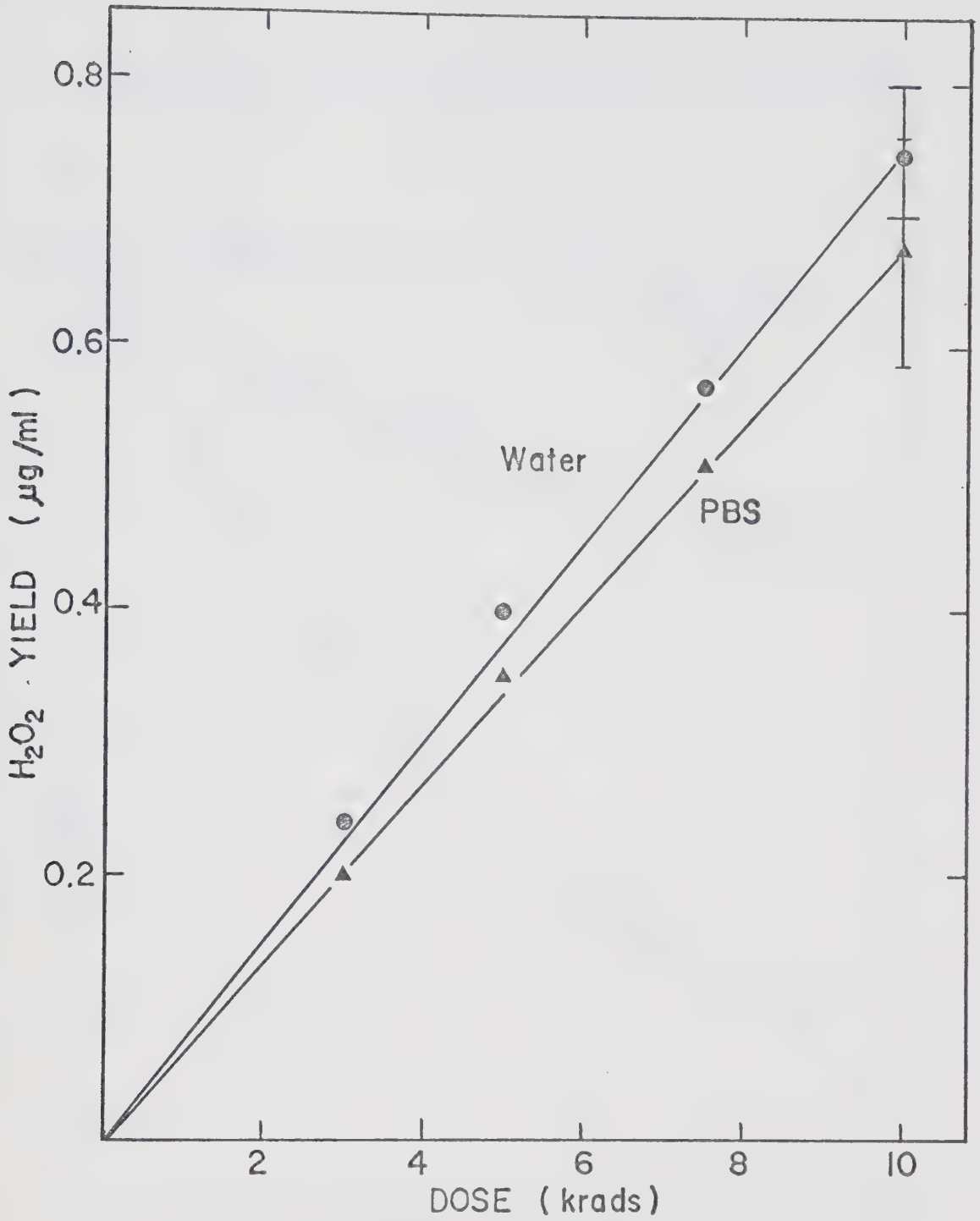


Figure 19. H_2O_2 yield measured by the starch-iodide method in irradiated water and PBS.

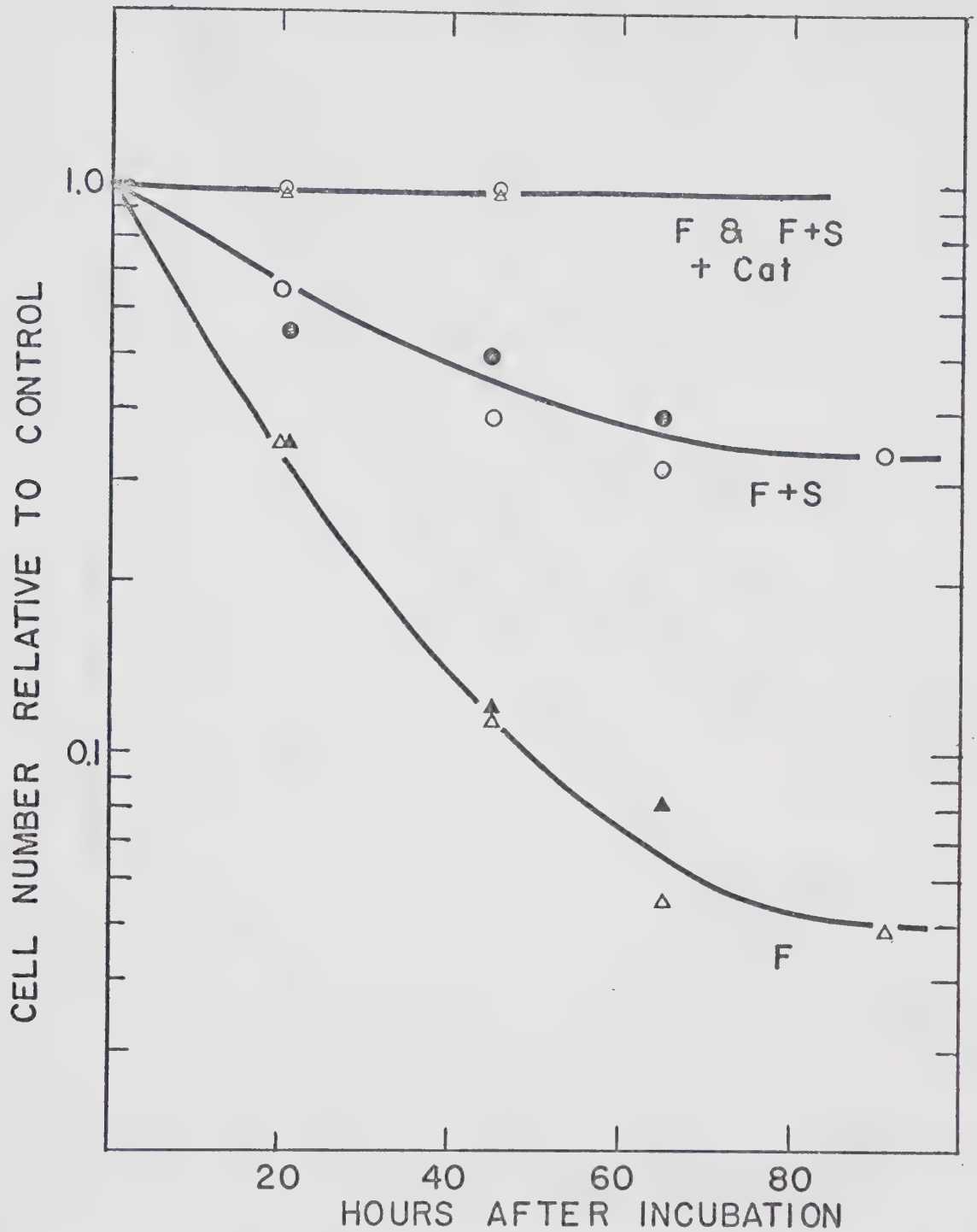


Figure 20. Comparison of post-incubation growth following exposure to irradiated medium (10 krad) and medium containing H_2O_2 (0.65 µg/ml).

a) Incubation in whole (F+S) and defined (F) media. Solid symbols represent growth after incubation with H_2O_2, open symbols present growth after incubation in irradiated medium. Catalase added before inoculation in irradiated medium offered complete protection (F & F+S + Cat).

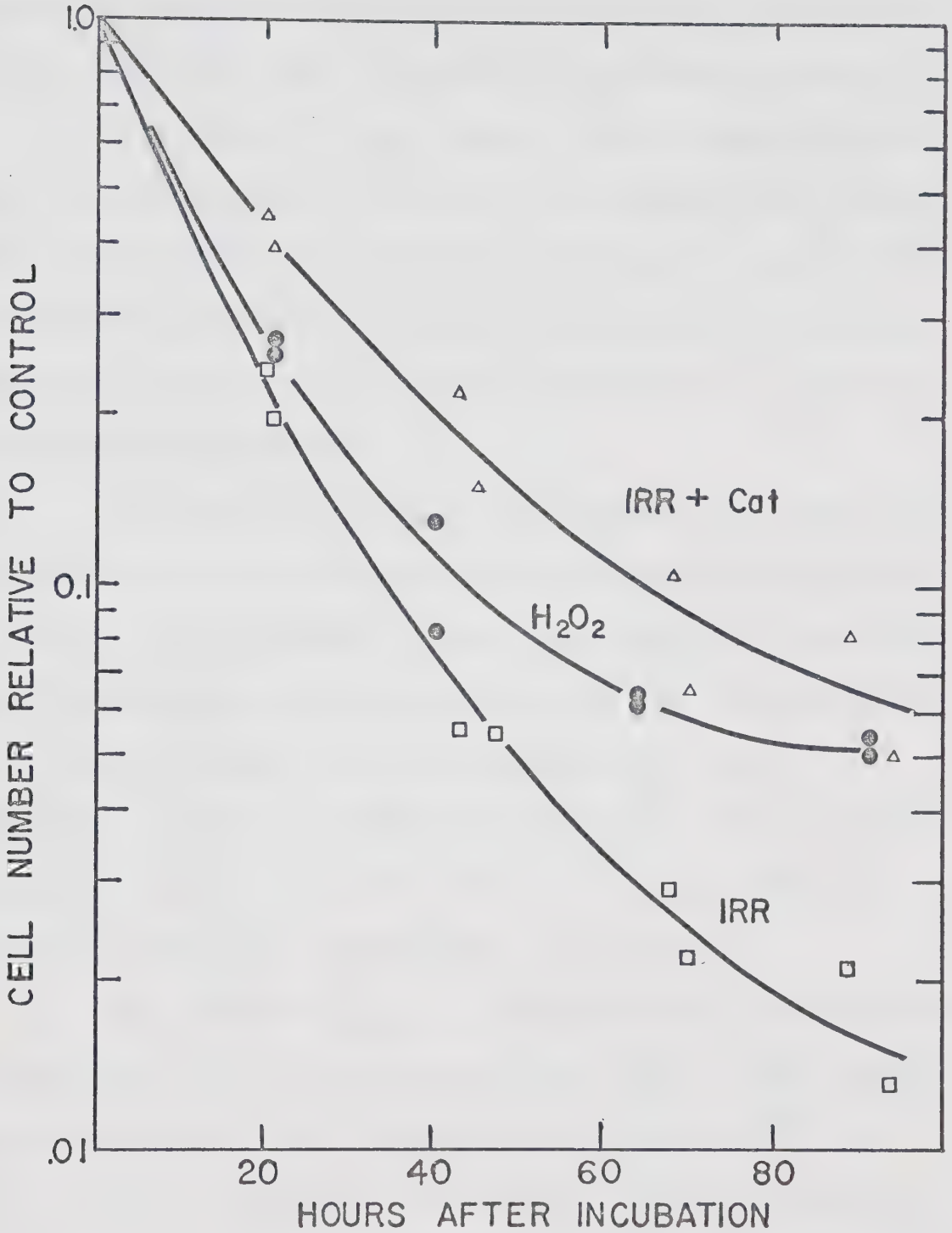


Figure 20b. Post incubation growth after incubation in irradiated PBS (IRR), or in PBS containing 0.8 μg/ml H_2O_2. Catalase added to irradiated PBS before inoculation offers only limited protection (IRR + Cat).

radiation produced hydrogen peroxide is solely responsible for the cytotoxic effect observed in irradiated medium.

The effect of H_2O_2 however, cannot describe the damage incurred during incubation in irradiated PBS (figure 20b). Furthermore, the addition of catalase 1 minute before inoculation offers only limited protection (cf. Table V1). These facts point clearly to the participation of some other radiation-formed species.

The mode of action of this species (we refer to it as singular, for convenience) must be direct, acting either as itself or as a precursor; the possibility mentioned above that the damaging action is due to inhibition of catalase protection is eliminated by the inability of H_2O_2 treatment to mimic the effect of irradiated medium, and by the data in table V11, which show that catalase added to irradiated PBS is not significantly inhibited.

The yield of H_2O_2 in irradiated PBS is approximately 20% less than that in irradiated water (figure 19). Since PBS contains 0.14 M Cl^-, compared to 0.012 M HPO_4^{2-} and 0.002 M $H_2PO_4^-$, the radiation chemistry which accounts for the different H_2O_2 yield undoubtedly involves the chloride ion. Determinations of H_2O_2 in irradiated aqueous solutions indicate that the H_2O_2 yield in PBS and 0.9% NaCl solution (0.15M Cl^-) was comparable, and that the "loss" of H_2O_2 in

TABLE VII

Catalase activity in irradiated aqueous solutions

	H_2O_2 Concentration (μg/ml)			
	H_2O_2	Irradiated H_2O_2 (10^2 krad)	Irradiated PBS (10 krad)	Expected *
Before catalase addition	0.57	0.76	0.63	
1 minute after catalase addition	0.15	0.16	0.19	
Time for 63% H_2O_2 digestion (min)	0.70	0.65	0.8	1.0

* based on catalase activity assay of Sigma

irradiated saline compared to water was not due to scavenging by Cl^- (Table VIII A). Subsequent measurements of H_2O_2 in irradiated solutions of different Cl^- concentrations revealed an increase of "lost" H_2O_2 with Cl^- concentration (Table VIII B). These data suggest that some radiation-chemical product of the chloride ion is formed in irradiated PBS.

TABLE VIII

H_2O_2 yield in irradiated aqueous solutions (μg/ml/10 krad)

A

H_2O	H_2O Concentrated NaCl added after irradiation to 0.15 M Cl^-	PBS 0.14 M Cl^-	0.9% NaCl 0.15 M Cl^-
0.76	0.76	0.64	0.63

B

H_2O	0.09% NaCl 0.015 M Cl^-	0.9% NaCl 0.15 M Cl^-	9% NaCl 1.54 M Cl^-
0.69	0.69	0.57	0.24

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 CELLULAR RADIOSENSITIVITY

Irradiated L5178Y cells exhibited a radiosensitivity typical of cultured mammalian cells. Their survival curve (figure 9), determined by agar colony formation, has a shoulder in the low dose region (n=2.0), and an exponential portion at higher doses (D_o=100 rads). Post-irradiation growth (figure 10) is such that virtually all cells, at survivals greater than about 10^{-3}, divide at least once after irradiation, indicating a period of latency between exposure and death.

The radiosensitivity of sub-cellular components was investigated by analysing the sucrose gradient sedimentation profiles of ribosomes irradiated *in situ* at moderate (500 rads) and high (3 krad) doses. No ribosomal degradation, nor shift in ribosome/polysome population was detected in either case.

5.2 EFFECTS OF INCUBATION IN IRRADIATED MEDIUM

Most of the present work was concerned with the delayed indirect effect of radiation on cells in culture. This was studied by exposing unirradiated cells to irradiated growth medium (Fischer's medium +10% horse serum).

Some toxic agent produced by irradiation of the

medium was shown to be responsible for appreciable cell death after short-term incubation at 37°C (figure 15), and incubation at 0°C. For both of these conditions any possible radiation-induced depletion of the nutritive capability of the medium would have no effect.

Addition of catalase to the irradiated medium 1 minute before inoculation offered total protection (Table Vl b); this was taken as evidence that peroxide was the radiation-produced cytotoxic agent. The rapidity of protection by catalase suggested reaction with hydrogen peroxide (bimolecular rate constant for formation of the catalase-H_2O_2 complex, $k = 3 \times 10^7\ M^{-1}sec^{-1}$), rather than a mono-substituted organic peroxide ($k = 0.9 \times 10^6 M^{-1}sec^{-1}$ for reaction with methyl hydrogen peroxide) (Chance 1949). Further evidence implicating H_2O_2 was obtained by incubating cells in medium containing H_2O_2 added according to the amount measured in irradiated water by the starch-iodide method (figure 19). The ability of reagent H_2O_2 to mimic the effect of irradiated medium is shown in figure 20(a). Finally, the time course of action of reagent H_2O_2 (figure 18) is similar to that seen in irradiated medium (figure 15b) Levinson (1966) also concluded that H_2O_2 was the damaging agent in irradiated Medium 199.

Berry et al. (1965) and Schubert et al. (1967)

reported that irradiated carbohydrate solutions retained their cytotoxic properties after months of storage, while Scott et al. (1966) and Szumiel et al. (1971) observed that the effect in culture medium had largely disappeared one day after irradiation. Results in figure 14 are consistant with the latter observation. (The different results are probably due to the 1000-fold difference in dose, Mrads vs. krads). The rate of increase in survival with inoculation time depended on the serum batch, and no increase was seen in irradiated PBS (figure 16). Furthermore, the extent of cell killing, determined by agar colony formation after incubation in irradiated medium inoculated at 1 minute varied widely (an effect also mentioned by Scott et al. 1966), and correlated with the rate of increase in survival (figure 16). These results suggest that the H_2O_2 formed in the medium is degraded or inactivated after irradiation by reaction with some serum component, and that the "anti-H_2O_2 activity", which varies from batch to batch, determines the survival and inoculation time dependence. The lack of an inoculation time dependence in PBS indicates that spontaneous degradation of H_2O_2 (e.g. hydrolysis) is comparatively unimportant. The serum mediated activity against H_2O_2 may be chemical or enzymatic. In this regard, it is interesting to note that Perlmann and Lipmann (1945) measured the amount of catalase

in human serum as 20 μg catalase/100 ml, and found that this value increased proportionately with the extent of hemolysis in the collected serum. Medium containing 10% serum with 20 μg/100 ml catalase will degrade half the initial H_2O_2 in 10 minutes.

Consistent with the idea that serum protects by inactivating H_2O_2 during and after irradiation was the observation (figure 20a) that incubation in defined medium is more damaging than in whole medium. That the effect was duplicated by reagent H_2O_2 and eliminated after addition of catalase showed that, as with whole medium, the cytotoxic agent in irradiated serumless medium was H_2O_2.

When cells were incubated in irradiated PBS in order to clarify the nature of the damaging species in a simple saline solution, it was found that added catalase offered significant, but not total protection (Table V1B). Because PBS is a purely ionic medium, the non-catalase protectable effect can not be due to mono- and di- substituted alkyl peroxides (which react slowly with catalase), found by Schubert and Watson (1969) in irradiated sucrose. Furthermore, the effect was "genuine", i.e. it was not due to catalase inhibition (Table V11). The "non-protectable" cytotoxic effect, therefore, must be due to a radiation product of one (or more) of the PBS anions, viz. Cl^-, $H_2PO_4^-$ and HPO_4^{2-}.

Measurements of H_2O_2 yield in NaCl solutions suggested the involvement of products of Cl^- (Table VIII).

The radiation-induced formation and subsequent chemistry of the Cl_2^- radical ion was discussed in Chapter 3. Conceivably, products formed in irradiated PBS resulting from Cl_2^- as precursor are Cl_2 (and possibly HOCl which is a hydrolysis product of Cl_2), and products of reaction between Cl_2^- and H_2O_2. These products are all oxidizing agents, and may be expected to have cytotoxic properties.

Phosphates in mM concentrations have been shown to enhance indirect effects of radiation (see e.g. Pollard and Weller 1967), and may also be responsible, or partly responsible for the "non-protectable" effect. This possibility was not investigated.

In summary, then, the scheme that is suggested by this work is that radiation-produced hydrogen peroxide is responsible for the cytotoxic effect originating in irradiated medium, and that the effect progressively decreases in the presence of serum. The protective effect of serum is attributed to degradation of H_2O_2 by reaction with serum proteins, either in a non-specific or enzymatic (catalatic) manner. The great variability in cellular response to irradiated medium can be explained by the presence of varying degrees of anti- H_2O_2 activity in the serum.

The more potent cytotoxic effect seen in irradiated PBS is due to H_2O_2 as well as other radiation-induced agents, likely products of the Cl^-_2 radical ion, formed by reaction of ·OH with Cl^- in the absence of more efficient ·OH scavengers.

The nature of the cellular damage was not studied. However, disintegrating cells were seen as soon as several hours after incubation, and this observation distinguishes medium-mediated damage from that due to direct irradiation. It is also consistant with the results of Levinson (1966) and Rosenberg and Matthews (1972) which indirectly and directly pointed to membrane damage as the primary site of the damaging action in irradiated medium.

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APPENDIX I

MEDIA FORMULATIONS AND COMPOSITION

1. Growth medium

a) Fischer's medium 90% (from GIBCO catalogue)

	mg/L		mg/L
NaCl	8000	threonine	30
$MgCl_2$	100	tryptophan	10
$NaH_2PO_4 \cdot H_2O$	69	tyrosine	60
KCl	400	valine	70
$Na_2HPO_4 \cdot 7H_2O$	113	asparagine	10
$CaCl_2 \cdot 2H_2O$	91	serine	15
glucose	1000	biotin	0.01
arginine	15	choline Cl	1.5
cystine	20	folic acid	10
glutamine	204	i-inositol	1.5
histidine HCl	60	nicotinamide	0.5
isoleucine	75	Ca pantothenate	0.5
leucine	30	pyridoxal HCl	0.5
lysine HCl	50	riboflavin	0.5
methionine	100	thiamine	1.0
phenylalanine	60	$NaHCO_3$	1125

b) Horse Serum 10% (from Altman and Dittmer 1961)

	g/100ml
Protein (total)	8
lipoprotein	0.5
glycoprotein	2
enzymes	
hormones	
Lipids (total)	0.6
Carbohydrates (total)	0.5
Non-protein nitrogenous substances	0.07
urea	0.05
amino-acids	0.03
Electrolytes	
Vitamins	

c) Antibiotics

Penicillin	100 units/ml
Streptomycin	100 µg/ml

2. Phosphate buffered saline (from GIBCO catalogue)

	mg/L	
NaCl	8000	
KCl	200	
Na_2HPO_4	1150	
KH_2PO_4	200	
$CaCl_2$ (anhydro)	100	
$MgCl_2.6H_2O$	100	
penicillin	100	units/ml
streptomycin	100	µg/ml